HORMONES AND STEROID METABOLISM BY RAT MAMMARY TUMOURS

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Declaration

I declare that I have composed this thesis myself and that it contains an accurate account of my own work.

Abstract

The hormonal control of 5α-reductase activity in DMBA (7,12-dimethylbenz[a]anthracene)- induced rat mammary tumours and its relationship to tumour growth have been studied by measuring tumour growth, tumour metabolism of testosterone and plasma hormone levels in rats of differing endocrine status.

Female Sprague-Dawley rats bearing actively growing tumours, induced by an intragastric administration of 30 mg DMBA at 50 days of age, were allocated to one of the following treatment groups:- 1) Intact Control (IC), 2) Intact + Perphenazine (IP), 3) Intact + Bromocryptine methanesulphonate (IB), 4) Ovariectomised Control (OC), 5) Ovariectomised + Perphenazine (OP). Animals received a daily s.c. dose of drug (5 mg/kg body weight) or corn oil vehicle (controls), over an average treatment period of 12 days. The effects of the treatment regimes on plasma prolactin and plasma oestradiol levels were measured in the individual tumour-bearing rats and also in greater detail in non-tumour-bearing rats. At the end of treatment (dioestrus in cycling rats), a whole tissue homogenate of tumour was incubated under fixed conditions with [7α-^{3}H] testosterone in the presence of an NADPH-generating system. 5α-Reduction was determined as the sum of 5α-dihydrotestosterone and total 5α-androstanediol production. Plasma prolactin levels were raised to the same extent in both IP and OP groups and were lowered in IB and OC groups. Plasma oestradiol levels were lowered in the OC, OP and IP groups, but not the IB group. The OC, OP and IP groups were in

constant dioestrus, whereas rats in the IB group cycled normally. Tumour growth was, on average, inhibited in the IB group and tumours regressed rapidly in the OC group. Tumour growth was stimulated in the IP group, but, in general, was not maintained in the OP group. In comparison with the IC group tumour 5α-reduction was significantly higher in the IP group, unaltered in the IB group, and numerically but not significantly lower in the OC group. Whilst tumour 5α-reductase in the OP group was significantly higher than that of the OC group it did not differ significantly from that of the IC group and was significantly less than that of the IP group. Although, in comparison with their respective control groups, the groups with elevated plasma prolactin levels showed a higher capacity for tumour 5α-reduction, other intergroup comparisons indicate that ovarian hormones may also be involved in regulating tumour 5α-reductase activity. A study of testosterone metabolism in actively growing DMBA-induced mammary tumours, taken from untreated rats at different stages of a 4-day oestrous cycle, revealed that 5α-reductase was highest in tumours removed in metoestrus and lowest in tumours removed in proestrus. However the differences in 5α-reduction levels between tumours from the 4 stages of the cycle did not attain significance. Evidence is presented to indicate that DMBA-induced rat mammary tumours convert testosterone to 4-androstene-3α,17β-diol. The daily s.c. administration (1 mg/rat) of 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol but not 5α-androstane-

3β,17β-diol induced a state of constant dioestrus and regression of DMBA-induced rat mammary tumours. The effects may be associated with a decreased secretion of oestradiol and prolactin. Tumour incidence was higher in rats given DMBA in proestrus and lower in those given DMBA in dioestrus. At all stages of the cycle the mean plasma prolactin levels at the time of DMBA administration were higher in rats which subsequently bore tumours than in those which did not.

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NOMENCLATURE - ABBREVIATIONS

Common and systematic names of steroids referred to in text

Common name and abbreviation	Systematic name
C-27	
cholesterol	5-cholestene-3β-ol
C-21	
corticosterone	11β,21-dihydroxy-4-pregnene-3,20,-dione
pregnenolone	3β-hydroxy-5-pregnene-20-one
progesterone	4-pregnene-3,20-dione
17α-hydroxyprogesterone	17α-hydroxy-4-pregnene-3,20-dione
C-19	
aetiocholanolone	3α-hydroxy-5β-androstane-17-one
5α-androstanediol (5α-Adiol)	refers to measurements made without separation of the 3,17-diol isomers
5α-androstanediol(3α) (5α-Adiol(3α))	5α-androstane-3α,17β-diol
5α-androstanediol(3β) (5α-Adiol(3β))	5α-androstane-3β,17β-diol
5α-androstanedione (5α-Adione)	5α-androstane-3,17-dione
Δ^4Diol(3α)	4-androstene-3α,17β-diol
Δ^4Diol(3β)	4-androstene-3β,17β-diol
5-androstenediol (Δ^5Diol)	5-androstene-3β,17β-diol

Common name and abbreviation	Systematic name
7α-hydroxy-5-androstenediol	5-androstene-3β,7α,17β-triol
16α-hydroxy-5-androstenediol	5-androstene-3β,16α,17β-triol
4-androstenedione (Δ^4Dione)	4-androstene-3,17-dione
androsterone	3α-hydroxy-5α-androstane-17-one
dehydroepiandrosterone (DHA)	3β-hydroxy-5-androstene-17-one
dehydroepiandrosterone sulphate ($DHASO_4$)	3β-sulphoxy-5-androstene-17-one
7α-hydroxy DHA	3β,7α-dihydroxy-5-androstene-17-one
7β-hydroxy DHA	3β,7β-dihydroxy-5-androstene-17-one
7-keto DHA	3β-hydroxy-5-androstene-7,17-one
16α-hydroxy DHA	3β,16α-dihydroxy-5-androstene-17-one
5α-dihydrotestosterone (5α-DHT)	17β-hydroxy-5α-androstane-3-one
5α-dihydrotestosterone acetate (5α-DHT acetate)	17β-acetyloxy-5α-androstane-3-one
testosterone (Testo)	17β-hydroxy-4-androstene-3-one
testosterone acetate (Testo acetate)	17β-acetyloxy-4-androstene-3-one
epitestosterone	17α-hydroxy-4-androstene-3-one

Common name and abbreviation	Systematic name
7α-hydroxytestosterone	7α,17β-hydroxy-4-androstene-3-one
16α-hydroxytestosterone	16α,17β-dihydroxy-4-androstene-3-one
C-18	
oestradiol (OE_2)	1,3,5(10)-estratriene-3,17β-diol
oestriol	1,3,5(10)-estratriene-3,16α,17β-triol
oestrone	3-hydroxy-1,3,5(10)-estratriene-17-one
oestrone sulphate	3-sulphoxy-1,3,5(10)-estratriene-17-one
2-methoxyoestrone	2-methoxy-3-hydroxy-1,3,5(10)-estratriene-17-one
2-methoxyoestrone sulphate	2-methoxy-3-sulphoxy-1,3,5(10)-estratriene-17-one

Abbreviations	Full names
ACTH	adrenocorticotrophic hormone
ave	average
CB 154 (B)	bromocryptine (2-bromo-12'-hydroxy-2'-(1-methylethyl) -5α- (2-methyl-propyl) ergotaman-3',6',18-trione) methanesulphonate
c.p.m.	counts per minute
d.f.	degrees of freedom
DMBA	7,12-dimethylbenz a anthracene
DNA	deoxyribonucleic acid(s)
d.p.m.	disintegrations per minute
g.l.c.	gas liquid chromatography
ln	logarithm to the base e
3-MC	3-methylcholanthrene
NADH	nicotinamide-adenine dinucleotide (reduced form)
$NADP^+$, NADPH	nicotinamide-adenine dinucleotide phosphate (oxidised and reduced forms)
perphenazine (P) (also Fentazin)	4- 3-(2-chlorophenothiazin-10-yl) propyl -1-piperazineethanol
5α-reductase	4-ene-3-ketosteroid,5α-oxido-reductase
RNA	ribonucleic acid(s)
r.p.m.	revolutions per minute
S.A.	specific activity
s.c.	subcutaneous(ly)
s.d.	standard deviation of observed values

Abbreviations	Full names
s.e. mean	standard error of estimate of mean values
t.l.c.	thin layer chromatography
u.v.	ultraviolet
v/v	volume by volume
w/v	weight by volume

Tumour positions

R	right
L	left
Nk	neck
Sh	shoulder
Ch	chest
Ax	axilla
Th	thorax
In	inguen
Gr	groin
An	anus

These names were used to denote the position of the tumours on palpation and do not necessarily correspond to particular mammary glands.

INTRODUCTION

I. Breast cancer

Early medical records suggest that cancer of the breast has occurred in the human species for at least 2,000 years (from Lewison, 1955). At present it is the most common form of malignancy in women of North America and Western Europe (Doll, Muir & Waterhouse, 1970; Campbell, 1971). The proportion of women in the UK and USA who contract the disease has been estimated at around 1 in 17 and 1 in 15 respectively (Forrest, 1971; Tormey & Carbone, 1976). The incidence of male breast cancer is less than 1% of that of females (Visfeldt & Scheike, 1973). Furthermore it has recently been reported that the incidence of breast cancer in women may be increasing (Grace, Gaudette & Burns, 1977; Devesa & Silverman, 1978), but the mortality rate due to the disease has not been markedly altered over the last three decades (Shimkin, 1967; Tormey & Carbone, 1976; Devesa & Silverman, 1978).

The study of breast cancer as a distinct form of malignancy has arisen from observations that, to varying degrees, tumours of the breast and subsequent metastases retain certain properties of their tissue of origin. Just as the development and growth of the "normal" female breast is under hormonal control (Lyons, 1958; Hilgers, 1971; Ceriani, Contesso & Nataf, 1972; Ceriani, 1974), evidence has accumulated to indicate that the development and growth of cancerous tumours within this tissue may be influenced by similar hormonal factors.

The distribution pattern of the world-wide incidence of breast cancer has been useful in studying the aetiology of the disease. Women in the USA and Western Europe exhibit a higher rate of breast cancer than women in other areas such as developing countries and Japan. There is some evidence that hormones may be involved in differences in breast cancer incidence between countries.

The markedly lower incidence of breast cancer found in postmenopausal Japanese women compared to their "western" counterparts (de Waard, 1964) has been associated with certain hormonal differences which exist between the two populations. MacMahon, Cole and Brown (1971) observed that high ratios of oestriol to oestrone plus oestradiol occur in the urine of Japanese women compared to American women. Based on this observation and the fact that early pregnancy, which protects against breast cancer, is associated with high oestriol excretion (Cole & MacMahon, 1969), it has been proposed that a lower risk of breast cancer is associated with an elevated excretion of oestriol (MacMahon, Cole, Brown, Aoki, Lin, Morgan & Woo, 1974). This theory has been criticised by Lipsett (1971) and Adams (1977), on the basis of the poor correlation between plasma and urinary oestrogen levels, and by Pratt and Longcope (1978), who were unable to find differences in oestriol production after oestradiol infusion in normal women and women with breast cancer. These criticisms have been further justified by the report that plasma levels of oestradiol and progesterone were unrelated to breast cancer risk in postmenopausal women (Bulbrook, Moore, Clark, Wang, Tong & Hayward, 1978).

Alternatively, Adams (1977) has proposed that the high levels of serum $DHASO_4$ and urinary androgen metabolites in British women compared to Japanese women are implicated in the higher incidence of postmenopausal breast cancer in western countries. This proposal is in alignment with the hypothesis that the "western" incidence of breast cancer is associated with overnutrition (de Waard, 1964). There is a strong world-wide correlation between breast cancer incidence and nutritional status, particularly fat intake (Lea, 1966; Drasar & Irving, 1973), and dietary factors, possibly involving fat, may contribute to the increase in breast cancer incidence towards "western" levels found amongst second generation Japanese born in the United States (Wynder, 1968; Buell, 1973). The levels of urinary androsterone and aetiocholanolone, both being important metabolites of DHA and $DHASO_4$ (van de Wiele & Lieberman,

1960), are high in obese women and are lowered during fasting as is the production rate of $DHASO_4$ (Borth, Linder & Riondel, 1957; Hendrikx, Heyns & de Moor, 1968). However it is also true that, as opposed to the situation in undeveloped areas, in the western countries of the world the menarche occurs earlier and first pregnancy later (Marshall, 1975), both factors being conducive to a higher incidence of breast cancer (MacMahon, Cole & Brown, 1973).

It might be argued that these results are equivocal because of the retrospective nature of the investigations. However, on the basis of a long term prospective study in Guernsey involving a population of approximately 5,000 women, apparently healthy at the start of the study, it has been demonstrated that premenopausal women with low excretory rates of androsterone and aetiocholanolone, which correlate with plasma levels of DHA, $DHASO_4$ and 5-androstenediol (Bulbrook, Hayward & Spicer, 1971; Wang, Moore, Thomas, Bulbrook, Hoare, Tong & Hayward, 1979), experience an increased risk of breast cancer.

The risk of breast cancer in premenopausal women also correlates with subnormal excretion levels of progesterone in the luteal phase of the menstrual cycle, but is unrelated to oestradiol levels (Bulbrook, Moore, Clark, Wang, Tong & Hayward, 1978). These findings agree with the suggestion of Wallace, Sherman, Bean, Leeper and Treloor (1978), that the increased breast cancer risk associated with early menarche, deferred pregnancy and late menopause (MacMahon, Cole & Brown, 1973; Juret, Couette & Mandard, 1976) may be due to a relative oestrogen excess unopposed by progesterone as reflected by longer and more varied cyclic patterns, at early and late stages of the reproductive period. Furthermore it has been reported that women undergoing hysterectomy and bilateral ovariectomy before the age of 40 had a reduced risk of contracting breast cancer (Trichopoulos, MacMahon & Cole, 1972; Hill, Wynder, Kumar, Helman, Rona and Kuno, 1976), and that the breast cancer incidence rate was

increased in postmenopausal women who received prolonged oestrogen replacement therapy (Hoover, Gray, Cole & MacMahon, 1976).

High prolactin levels have been found in association with a family history of breast cancer (Kwa, de Jong-Bakker, Engelsman & Cleton, 1974; Henderson, Gerkins, Rosario, Casagrande & Pike, 1975; Kwa, Cleton, de Jong-Bakker, Bulbrook, Hayward & Wang, 1976), but, in general, a lack of correlation between plasma prolactin levels and the presence or absence of breast cancer hasd been reported (Boyns, Cole, Griffiths, Roberts, Buchan, Wilson & Forrest, 1973; Sheth, Ranadive, Suraiya & Sheth, 1975; Franks, Ralphs, Seagroatt & Jacobs, 1974).

Despite the fact that the period of neoplastic change cannot yet be determined, present evidence would suggest that hormones may play a role in the development of breast cancer. The questions as to which hormones are important and whether, in the human, these hormones can act directly as carcinogens or whether their effect is to render the tissue more susceptible to as yet unidentified carcinogenic agents remain to be answered.

The first indication that the growth of established mammary cancers may be controlled in some cases by endocrine manipulation was provided as early as 1896 by Beatson who reported the regression of breast tumours in premenopausal women following ovariectomy. Later it was shown that adrenalectomy (Huggins & Bergenstal, 1952; Dao, 1972) and pituitary ablation (Luft & Olivecrona, 1953; Forrest & Stewart, 1967) were of benefit to a proportion of postmenopausal women with breast cancer. In addition certain groups of breast cancer patients have derived benefit from the administration of androgens (Co-operative Breast Cancer Group, 1964; Kennedy & Yarbro, 1968; Hayward, 1970), progesterone (Jolles, 1962; Briggs, Caldwell & Pitchford, 1967), large doses of oestrogens (Hayward, 1970; Stoll, 1973) and anti-oestrogens (Cole, Jones & Todd, 1971; EORTC, 1972; Ward, 1973). Amelioration of the disease has also been reported following treatment with drugs which interfere with the secretion of adrenal steroids such as aminoglutethimide (Wells, Haagensen, Misbin, Lipton, Smart &

Santen, 1976; Smith & Powles, 1978) and dexamethasone (Borkowski, L'Hermite, Dor, Longeval, Rozencweig, Muquardt & van Cauter, 1977).

Whilst certain breast cancers in experimental animals have been shown to be dependent upon circulating levels of prolactin (Welsch & Nagasawa, 1977), and a role for prolactin in human breast cancer has also been proposed (McGuire, Chamness, Costlow & Shepherd, 1974; Minton, 1974 a; Smithline, Sherman & Kolodny, 1975; Nagasawa, 1979), the use of drugs such as L-Dopa and CB 154 which inhibit prolactin release from the anterior pituitary have generally provided disappointing results in the treatment of breast cancer although remissions have been observed in a few individuals (Dickey & Minton, 1972; Heuson, Coune & Staquet, 1972; Murray, Mozaffarian & Pearson, 1972; Schulz, Czygan, del Pozo & Friesen, 1973; Minton, 1974 b).

Although some primary breast cancers and metastases retain their responsiveness to the hormones which regulate the growth and function of their tissue of origin, this property appears absent or is eventually lost by a large proportion of tumours. Since a positive response to hormone or ablative endocrine therapy is seen in less than half of the women presenting with breast cancer, it is important to be able to recognise these patients so that the most appropriate form of therapy can be given. Much effort has therefore been directed towards the use of in vitro methods to demonstrate hormonal sensitivity in excised tumours.

The discovery that oestrogen target tissues possess cytoplasmic and nuclear receptors exhibiting a high specificity and high affinity towards oestradiol (Talwar, Segal, Evans & Davidson, 1964; Toft & Gorski, 1966; Jungblut, Hatzel, de Sombre & Jensen, 1967) explained the earlier observation of oestrogen retention by target tissues (Glascock & Hoekstra, 1959; Jensen & Jacobsen, 1960 & 1962), and provided evidence that the action of steroids was mediated by attachment to specific receptors.

In keeping with the observation that some breast cancers exhibit sensitivity to oestrogens, appreciable levels of specific, high-affinity oestradiol receptors

have been found in the cytosol and nuclei prepared from breast cancers (Jensen, de Sombre & Jungblut, 1967; Terenius, 1968; Sander, 1969; Brooks, Locke & Soule, 1973). Moreover, the presence of specific cytosol and nuclear receptors for progesterone (Terenius, 1973; Horowitz & McGuire, 1975), androgens (Wagner, Gorlich & Jungblut, 1973; Maass, Engel, Trams, Nowakowski & Stolzenbach, 1975) and glucocorticoids (Teulings, Treurniet, Alexieva-Figusch, Blonk-van der Wijst & van Gilse, 1977), and cell membrane receptors for prolactin (Shiu, Kelly & Friesen, 1973; Costlow, Buschow & McGuire, 1974; Morgan, Raggatt, de Souza, Salih & Hobbs, 1977) and for insulin (Holdaway & Friesen, 1977) have also been identified in specimens of human breast cancer. In addition to oestrogen receptors, the MCF-7 cell line also possesses specific receptors for progesterone, androgens and glucocorticoids (Horowitz, Costlow & McGuire, 1975). The levels of the above-mentioned receptors vary greatly between specimens of breast cancer from different patients, with frequently negligible or unmeasureable levels being reported. From the follow-up data of several centres routinely measuring oestradiol receptor levels it can be concluded that although the presence of receptors is essential for, it is not necessarily proof of, hormone dependency in such cancers (McGuire, Carbone & Vollmer, 1975).

An alternative approach which is more elaborate but may give a better indication of functional hormone sensitivity is to study the influence of hormones on tumour explants maintained in organ culture or cell lines derived from breast cancer tissue. Parameters such as cell proliferation, DNA, RNA and protein synthesis and enzyme activity have been used to monitor the responses of these preparations. Using this technique, Hobbs and his group have claimed that explants of about half the tumours studied appear to be better preserved in the presence of prolactin, oestradiol or testosterone, either alone or in combination (Flax & Salih, 1973; Flax, Salih, Newton & Hobbs, 1973; Hobbs, de Souza & Salih, 1974). The stimulatory effect of prolactin and also that of insulin on

tumour explants has been confirmed (Dilley & Kister, 1975; Welsch & McManus, 1977), and stimulatory and inhibitory effects of both oestradiol and testosterone have been reported by other groups working on explants (Burstein & Carey, 1974; Finkelstein, Geier, Horn, Levij & Ever-Hadani, 1975). Cell lines from breast cancer also exhibit hormonal sensitivity, which appears to correlate with the presence of specific hormone receptors (Lippman, Bolan & Huff, 1976 a, b, c). The reports from a limited number of short follow-up studies claim that the tumour responses in vitro relate to clinical responses (Salih, Flax & Hobbs, 1972; Flax, Salih, Newton & Hobbs, 1973; Burstein & Carey, 1974).

In addition to the presence of receptors and hormonal sensitivity of explants in vitro, a property of breast cancer tissue which is of importance in considerations of hormone dependency or sensitivity is its ability to metabolise steroid hormones. Breast cancer preparations have been found capable of the synthesis, from relatively inert precursors, of both hormonally active steroids and their subsequent metabolic inactivation. The former mechanism, because of its close resemblance to those described in the classical endocrine organs, has earned breast cancer the description of para-endocrine (Adams & Wong, 1968 a). The steroid interconversions known to occur in breast cancer tissue are described in greater detail later in the INTRODUCTION as this subject forms the basis of the present studies. Of particular interest was the effect of hormonal manipulation on tumour growth in relation to tumour metabolism of androgens, a group of steroids reported to be capable of inhibiting the growth of breast cancer.

Since it would be unethical to raise the levels of certain hormones in patients with breast cancer, an animal model has been chosen for the study of the interrelationships between hormone levels, mammary tumour growth and androgen metabolism. This approach has the advantages that, in animals with a common background living in a controlled environment, one can obtain a large number of rapidly growing tumours whose growth can be easily and accurately

monitored. In addition treatment regimes can be adopted which increase tumour growth rate. The inevitable disadvantage of this approach is that extrapolation of results from one species to another has many restrictions, but useful basic knowledge can be acquired providing the model is appropriate for the parameters under study.

Unlike the human species breast cancer is relatively rare in other species with the exception of the domestic dog (Schneider, 1970), cat (Dorn, Taylor, Schneider, Hibbard & Klauber, 1968) and certain laboratory strains of mouse, rat and rabbit (Gardner, 1953; Hamilton, 1974; Short & Driffe, 1977). Since breast cancer is also rare in the wild counterparts of these species (Hamilton, 1974), it appears that the increased rate of breast cancer may be due to the artificial restrictions imposed by man possibly associated with breeding restraints (Short & Driffe, 1977; Dorn & Schneider, 1976). The incidence of spontaneous mammary cancer in cats, dogs and rabbits is too low for use as experimental models, and the deliberate induction of tumours in these species for research purposes does not appear to have been investigated. The two species commonly used for large scale research purposes are the mouse and rat.

Although the spontaneous incidence of breast cancer in certain strains of mice is almost 100% (Nandi & McGrath, 1973), the tumours take a considerable time to appear and the growth of advanced tumours is not usually hormone dependent (de Ome, Bern & Elias, 1958; Mühlbock, 1972). Moreover viral components are strongly implicated in the aetiology of mammary tumours in mice (Bittner, 1936), but their possible involvement in human breast cancer is still unclear.

Alternatively, under favourable conditions in the rat, oestrogenic compounds, such as oestrone (McEuen, 1938; Geschickter & Byrnes, 1942; Cutts & Noble, 1964) and diethylstilbestrol (Nelson, 1944; Dunning, Curtis & Segaloff, 1947), synthetic chemical carcinogens, such as 2-acetaminofluorene (2AAF) (Wilson, de Eds & Cox, 1941; Bielschowsky, 1944), 3-methylcholanthrene (3-MC)

(Dunning, 1940; Shay, Aegerter, Gruenstein & Komarov, 1949) and 7, 12-dimethylbenz(a)anthracene (DMBA) (Geyer, Bleish, Bryant, Robbins, Saslaw & Stare, 1951) and various forms of radiation (Huggins & Fukunishi, 1963) rapidly induce tumours of the mammary gland which are largely hormone dependent (Dao, 1964).

II. The DMBA-induced mammary tumour model

The mammary tumour model adapted for the present work was that induced by DMBA in the female rat. Under optimal conditions, every rat given DMBA will develop mammary tumours. These tumours have much in common with human breast cancer with regard to the involvement of endocrine factors on induction, site of origin, histological appearance and influence of hormones on tumour growth. For these reasons this model has been widely studied. It is thus pertinent to outline some of the properties of the DMBA-induced tumour, drawing comparisons with the human disease where relevant.

Chemical induction of breast tumours was first observed by Maisin and Coolen (1936), following the repeated application of 3-MC to the skin of mice. 3-MC was also found to induce mammary tumours in rats (Shay, Aegerter, Gruenstein & Komarov, 1949). The synthesis of DMBA (Bachmann & Chemerda, 1938; Newman, 1938) provided an alternative carcinogen for the induction of mammary tumours (Geyer, Bleish, Bryant, Robbins, Saslaw & Stare, 1951), which proved more potent that 3-MC (Huggins, Grand & Brillantes, 1961). Huggins, Grand and Brillantes (1961) reported no fatalaties and the appearance of mammary tumours in 100% of a series of female Sprague-Dawley rats given a single oral dose of DMBA at 50 days of age. Although DMBA is the more potent carcinogen, 3-MC has been used for several mammary tumour studies. Since the properties of tumours induced by the two carcinogens appear to be identical, work carried out on not only DMBA - but also 3-MC-induced mammary tumours is cited.

It has been shown that the induction of mammary tumours is highly dependent on the strain of rat, with Sprague-Dawley appearing to be the most susceptible (Kim & Furth, 1960 a; Boyland & Sydnor, 1962; Sydnor, Butenandt, Brillantes & Huggins, 1962; Engelbart & Gericke, 1964; Gruenstein, Meranze, Thatcher & Shimkin, 1966). The age at which the carcinogen, whether 3-MC or

DMBA, is given is also a critical factor in mammary tumour induction, the most susceptible period in Sprague-Dawley females being 50 - 60 days of age (Huggins, Grand & Brillantes, 1961; Dao, 1969). There is strong evidence that DMBA acts directly on the mammary gland to induce tumours (Dao, Tanaka & Gawlak, 1964; Brennan, Grau & Singley, 1966; Dao, King & Gawlak, 1968; Dao & Sinha, 1972; Sinha & Dao, 1974), and it has been postulated that the stage and rate of development of the breast is critical with regard to these variations in susceptibility (Huggins, Grand & Brillantes, 1959; Dao, 1965 and 1969; Russo & Russo, 1978 a). As the breast develops under hormonal regulation the level of DNA, the rate of DNA synthesis, and the binding of DMBA to the DNA of epithelial cells correlate well with susceptibility to mammary tumour induction (Janss, Moon & Irving, 1972; Nagasawa & Yanai, 1974; Fysh & Okey, 1978; Janss & Ben, 1978; Russo & Russo, 1978 b). It is generally accepted that DMBA, like most other carcinogens, must be metabolically activated to form covalent bonds and exert a carcinogenic effect (Sims & Grover, 1974; Heidelberger, 1975; Tamulski, Morreal & Dao, 1973; de Pierre & Ernster, 1978).

Since the development of the breast and the associated rate of epithelial cell DNA synthesis are hormonally controlled (Lyons, 1958; Lyons, Li & Johnson, 1958; Nagasawa & Yanai, 1974; Stoudemire, Stumpf & Sar, 1975), it is not surprising that in common with breast cancer in humans, endocrine factors are also involved in the induction of mammary tumours in the rat by carcinogens. Thus early pregnancy (Dao, Bock & Greiner, 1960), and prior ovariectomy (Dao, 1962 and 1968; Welsch, Clemens & Meites, 1968) prevented the occurrence of the chemically induced tumours. However if ovariectomy was performed slightly before carcinogen treatment, concurrent treatment with prolactin and growth hormone increased tumour incidence significantly compared to ovariectomised controls (Talwalker, Meites & Mizuno, 1964). In contrast, when ovariectomy was performed 30 days prior to carcinogen treatment elevation of prolactin secretion did not increase incidence (Welsch, Clemens & Meites, 1968).

The inhibitory effect of ovariectomy on tumour induction was overcome by concommitant administration of progesterone and low, but not high, doses of oestradiol (Huggins, Briziarelli & Sutton, 1959). Early administration of oestrogen (Huggins, 1965) and progesterone (Welsch, Clemens & Meites, 1968) protected the mammary epithelium against the carcinogenic action of DMBA.

Whilst the absolute requirement for ovarian hormones at the time of induction is unclear, the role of prolactin appears vital. Mammary tumour induction by 3-MC or DMBA fell sharply when plasma prolactin levels were reduced by hypophysectomy (Huggins, Grand & Brillantes, 1959), and by ergot alkaloids (Clemens & Shaar, 1972; Nagasawa & Yanai, 1976). Tumour incidence was also decreased in various conditions associated with hyperprolactinaemia prior to carcinogen administration such as pregnancy (Dao, Bock & Greiner, 1960), pituitary homografts (Welsch, Clemens & Meites, 1968), hypothalamic lesions (Welsch, Clemens & Meites, 1969; Klaiber, Gruenstein, Meranze & Shimkin, 1969), ether stress (Gala & Loginsky, 1973), reserpine (Welsch & Meites, 1970), oestrogens (Kledzik, Bradley & Meites, 1974) and oral contraceptives (Weisburger, Weisburger, Griswold & Casey, 1968; Welsch & Meites, 1969).

The role of prolactin in mammary carcinogenesis is not simple, and it appears that the level of prolactin is important in the control of the developmental stage of the breast at which time the tissue is most susceptible to carcinogenic attack (Meites & Clemens, 1972; Meites, Lu, Wuttke, Welsch, Nagasawa & Quadri, 1972). If prolactin levels are within the normal range the breast is highly susceptible to carcinogens between 50 and 60 days of age (Huggins, Grand & Brillantes, 1961; Dao, 1969). By abnormally elevating plasma prolactin two or three weeks prior to carcinogen administration the development of the breast is accelerated leaving the breast refractory to tumour induction by 50 days of age (Nagasawa & Yanai, 1974). Conversely a reduction in prolactin levels delays or inhibits normal mammary gland development and thus reduces susceptibility (Nagasawa & Yanai, 1976).

DMBA-induced tumours appear to arise from the intraductal proliferation of the epithelial cells of ducts or terminal end bud elements (Middleton, 1965; Moon, 1969; Murad & von Haam, 1972; Russo, Saby & Isenberg, 1976; Russo, Saby, Isenberg & Russo, 1977). The high level of terminal end buds differentiating at the age when rats are most susceptible to mammary carcinogens correlates with tumour incidence and DNA synthesis (Russo & Russo, 1978 a & b). It has been postulated that the majority of human breast cancers are also ductal in origin (Muir, 1941; Wellings, Jensen & Marcum, 1975). Almost all tumours appearing early after DMBA treatment are classified as adenocarcinomas and histologically resemble the adenocarcinomas commonly found in human breast cancer (Young, Cowan & Sutherland, 1963; Dao, 1964; Murad & von Haam, 1972; Russo, Saby, Isenberg & Russo, 1977). Within this general classification, variations in mitotic indices, differentiation and structural elements may be observed (Murad & von Haam, 1972; Young & Hallowes, 1973). Considerable variations may occur between tumours on the same rat and between different areas of a single tumour (Huggins, Briziarelli & Sutton, 1959; Young & Hallowes, 1973). Mammary fibroadenomas and nodular adenoses may also be induced by DMBA but these, in general, have a longer latent period than the adenocarcinomas (Gruenstein, Meranze, Thatcher & Shimkin, 1966).

Adenocarcinomas induced by DMBA appear very rapidly (two - three months) after administration of the carcinogen and grow quickly (Huggins, Grand & Brillantes, 1961; Huggins, Moon & Morii, 1962; Huggins & Yang, 1962). Multiple primary tumours are very common (Huggins, Grand & Brillantes, 1961), but metastases are rare, although local invasion of surrounding tissue such as skin and muscle may occur (Huggins, Briziarelli & Sutton, 1959; Young & Cowan, 1963). Lack of metastases contrasts sharply with human breast cancer, but it has been reported that splenectomy or thymectomy or both permit metastases of the DMBA-induced tumour (Kim, 1970).

Not all adenocarcinomas induced by DMBA continue to grow with age, and some will become static or regress (Young, Cowan & Sutherland, 1963; Heise & Gorlich, 1966). Reports vary as to the proportions of static and regressing tumours, but it appears that the percentage of actively growing tumours reduces with age (Bradley, Kledzik & Meites, 1976). Rapid regrowth of some regressing and static tumours can occur after a time interval (Heise & Gorlich, 1966). All three growth variants can be found in individual rats with multiple tumours and have been reported to occur independently of the prevailing hormonal state or tumour histology (Young & Cowan, 1963). Other workers have reported a correlation between spontaneous growth and the histological appearance of tumours (Stevens, Stevens & Currie, 1965). It has been proposed that the variations in growth may be due to immunological factors possibly associated with the adrenal necrosis induced by DMBA (Huggins & Morii, 1961; Young & Cowan, 1963; Heise & Gorlich, 1966).

Just as hormones play an important role in the initiation of mammary carcinogenesis by DMBA, the hormonal status of the animal plays a profound role in the development and growth of these mammary cancers. Hypophysectomy or ovariectomy performed after DMBA administration will cause a decrease in the number of palpable tumours per rat and will cause regression in the majority of growing tumours (Dao, 1959; Huggins, Briziarelli & Sutton, 1959; Daniel & Prichard, 1963; Young, Cowan & Sutherland, 1963). Marked tumour regression also occurs after combined ovariectomy and adrenalectomy (Sterental, Dominguez, Weissman & Pearson, 1963; Nagasawa & Yanai, 1970), but adrenalectomy alone has a stimulatory effect on tumour growth (Chen, Bradley & Meites, 1976). As with human breast cancer the responses of individual DMBA-induced tumours to hormonal manipulation showed marked variation (Segaloff, 1968; Nagasawa, Chen & Meites, 1973; Bradley, Kledzik & Meites, 1976), but a far greater proportion of the carcinogen-induced tumours are hormone dependent than their human counterparts (Young, Cowan & Sutherland,

1963; Daniel & Prichard, 1963; Dao, 1964). It has been reported that the hormonal dependency of DMBA-induced tumour decreases with age (Griswold & Green, 1970; Bradley, Kledzik & Meites, 1976).

From a wide range of experimental evidence it is accepted that the key pituitary hormone controlling the development and growth of DMBA-induced mammary tumours is prolactin. Thus a reduction in plasma prolactin levels whether by hypophysectomy (Daniel & Prichard, 1963; Sterental, Dominguez, Weissman & Pearson, 1963; Pearson, Llerena, Llerena, Molina & Butler, 1969), anti-rat prolactin serum (Butler & Pearson, 1971) or specific drug treatment (Muckter, Frankus & More, 1970; Nagasawa & Meites, 1970; Heuson, Waelbroeck-van Gaver & Legros, 1970; Cassell, Meites & Welsch, 1971; Stahelin, Burckhardt-Vischer & Fluckiger, 1971; Meites, Lu, Wuttke, Welsch, Nagasawa & Quadri, 1972; Quadri, Clark & Meites, 1973; Quadri, Kledzik & Meites, 1973; Welsch, de Iturri & Meites, 1973; Chan & Cohen, 1974; Sweeney, Poore, Kornfeld, Bach, Owen & Clemens, 1975) reduced the number of palpable tumours and caused regression of growing tumours. Conversely an increase in the number of palpable tumours and an increase in the growth rate of established tumours were observed when plasma prolactin levels were elevated by a variety of procedures including adrenalectomy (Chen, Bradley & Meites, 1976), pregnancy (Dao & Sunderland, 1959; McCormick & Moon, 1965), implantation of pituitary homografts and pituitary tumours (Kim & Furth, 1960 a; Welsch, Clemens & Meites, 1968; Takizawa, 1973; Harada, 1976), hypothalamic lesions (Clemens, Welsch & Meites, 1968; Klaiber, Gruenstein, Meranze & Shimkin, 1969; Welsch, Clemens & Meites, 1969; Sinha, Cooper & Dao, 1973), hypothalamic implants of steroids (Nagasawa, Chen & Meites, 1969; Nagasawa & Meites, 1970), and specific drug treatment (Pearson, Llerena, Llerena, Molina & Butler, 1969; Meites, 1970; Meites, Lu, Wuttke, Welsch, Nagasawa & Quadri, 1972; Quadri, Kledzik & Meites, 1973). However there appears to be no correlation between serum prolactin levels that lie in the "normal range" and rats bearing

either fast-or slow-growing DMBA-induced tumours (Nagasawa, Chen & Meites, 1973).

Although the secretion of other hormones apart from prolactin may be changed by these procedures there is sufficient evidence to suggest that prolactin is important. The circulating levels of growth hormone may also be altered by some of the treatments listed above. However the median eminence lesions, which stimulate tumour growth, raise prolactin output but depress growth hormone secretion (Klaiber, Gruenstein, Meranze & Shimkin, 1969; Welsch, Clemens & Meites, 1969; Sinha, Cooper & Dao, 1973). Anti-rat prolactin which causes tumour regression is specific for prolactin and has little crossover reaction with growth hormone (Butler & Pearson, 1971). Furthermore the administration of prolactin free of growth hormone caused a stimulation of tumour growth (Meites, 1972), and reversed the regression imposed by hypophysectomy (Pearson, Llerena, Llerena, Molina & Butler, 1969). Administration of growth hormone itself has been reported to have no effect (Pearson, Llerena, Llerena, Molina & Butler, 1969; Nagasawa & Yanai, 1970), or a slightly stimulatory effect (Li & Yang, 1974) on the growth of DMBA-induced tumours.

Immediate regression has been reported to occur in 75 - 90% of DMBA-induced tumours in rats following ovariectomy (Young, Cowan & Sutherland, 1963; Teller, Kaufman, Bowie & Stock, 1969; Bradley, Kledzik & Meites, 1976). Tumours often regrow when the ovariectomised rats are given progesterone plus low doses of oestradiol (Young & Cowan, 1963), or low doses of oestradiol alone (Sterental, Dominguez, Weissman & Pearson, 1963; Talwalker, Meites & Mizuno, 1964). Huggins, Briziarelli and Sutton (1959) observed a stimulation of mammary tumour growth in rats ovariectomised prior to DMBA and given only progesterone, but progesterone alone was insufficient to reverse the inhibitory effects of ovariectomy performed on tumour-bearing rats (Kelly, Asselin, Labrie & Raynaud, 1977). However it has also been reported that in untreated

ovariectomised rats certain tumours will regrow after a lengthy latent period (Boylan, Fowler & Wittliff, 1977).

Growth of DMBA-induced mammary tumours is also inhibited by high doses of oestradiol (Huggins & Yang, 1962; Dorfman, 1965), and anti-oestrogenic compounds, such as tamoxifen (ICI 46,474) (Nicholson & Golder, 1975), nafoxidine (U 11100 A) (Terenius, 1971; Heuson, Waelbroeck-van Gaver, Legros, Gallez, Robyn & L'Hermite, 1972), Parke-Davis CI 628 (de Sombre & Arbogast, 1974), and RU 16117 (Kelly, Asselin, Caron, Raynaud & Labrie, 1977). Large doses of prolactin overcome the inhibitory effect of oestradiol (Meites, Cassell & Clark, 1971), but the high doses of oestradiol do not inhibit prolactin secretion and appear to exert their effect by direct action at tumour level (Meites, Lu, Wuttke, Welsch, Nagasawa & Quadri, 1972).

The administration of progesterone (Huggins, Moon & Morii, 1962; Jabara & Harcourt, 1970; Kelly, Asselin, Labrie & Raynaud, 1977), low doses of oestrogens (Huggins, Grand & Brillantes, 1961), or the contraceptive pill combination, Enovid (Welsch & Meites, 1969), have all been reported to stimulate tumour growth in intact rats.

Since a decrease or increase in circulating oestrogen causes a corresponding change in prolactin levels (Nicoll & Meites, 1962 & 1964; Meites & Nicoll, 1966), there has been debate as to whether the effects of oestrogen are mediated through prolactin secretion as proposed by Furth (Furth, 1967 & 1972), or whether the tumour has a basic requirement for ovarian hormones (Dao, 1971). In an attempt to resolve this problem, tumour growth has been monitored in ovariectomised and ovariectomised-plus-adrenalectomised rats in which the levels of lactogenic hormone have been artificially maintained or raised (Pearson, Llerena, Llerena, Molina & Butler, 1969; Welsch, Clemens & Meites, 1969; Nagasawa & Yanai, 1970 & 1973; Sinha, Cooper & Dao, 1973; Leung, Sasaki & Leung, 1975). Under these conditions tumour growth continues or is in some cases stimulated for several days but these effects do not usually persist,

and most tumours eventually regress. Rapid regrowth could be obtained by grafting ovaries into such rats (Clemens, Welsch & Meites, 1968; Sinha, Cooper & Dao, 1973). However Pearson, Llerena, Llerena, Molina and Butler (1969) found that the administration of ovine prolactin to rats whose tumours had regressed completely following ovariectomy and adrenalectomy caused complete regrowth of tumours. In addition, prolonged growth of 3-MC-induced tumours in ovariectomised rats whose prolactin levels were grossly elevated by pituitary tumour grafts has been reported (Kim & Furth, 1960 b). Hormone profiles were not thoroughly studied and the role of the adrenal glands, or additional properties of the grafts, were not assessed. Whereas it appears that elevated prolactin levels delay the inhibitory effect of ovariectomy on tumour growth the administration of steroids to hypophysectomised rats did not prevent tumour regression (Sterental, Dominguez, Weissman & Pearson, 1963; Pearson, Llerena, Llerena, Molina & Butler, 1969). From these data it seems likely that the continued growth of most DMBA tumours has an absolute requirement for both pituitary and ovarian hormones acting in synergism (Sterental, Dominguez, Weissman & Pearson, 1963; Leung, Sasaki & Leung, 1975), but that growth rate is particularly sensitive to changes in prolactin levels (Bradley, Kledzik & Meites, 1976).

This concept has received support from the demonstration in DMBA-induced mammary tumours of not only specific cytoplasmic and nuclear receptor proteins for oestradiol (King, Cowan & Inman, 1965; Mobbs, 1966; Sander & Attramadal, 1968; Terenius, 1968; Kyser, 1970), and progesterone (Terenius, 1973; Asselin, Labrie, Kelly, Philibert & Raynaud, 1976; Koenders, Geurts-Moespot, Zolingen & Benraad, 1977), but also specific prolactin receptors in the cell membrane (Turkington, 1974; Kelly, Bradley, Shiu, Meites & Friesen, 1974; de Sombre, Kledzik, Marshall & Meites, 1976). Prolactin may influence the concentration of oestrogen receptors (Vignon & Rochefort, 1974 & 1976; Leung & Sasaki, 1975; Sasaki & Leung, 1975), and oestradiol the number of

receptor sites for prolactin binding (Kledzik, Bradley, Marshall, Campbell & Meites, 1976).

In addition to high doses of oestrogen and anti-oestrogens, the administration of naturally-occurring and synthetic androgens inhibit the growth of DMBA-induced mammary tumours (Huggins, Briziarelli & Sutton, 1959; Harada, Rooks & Dorfman, 1965; Griswold, Skipper, Laster, Wilcox & Schabel, 1966; Teller, Stock, Stohr, Merker, Kaufman, Escher & Bowie, 1966; Heise & Gorlich, 1966; Griswold & Green, 1970), and of a hormone-sensitive transplanted mammary fibroadenoma (Huggins & Mainzer, 1957). It is interesting that raising the dose of testosterone abolished the inhibitory effect of lower doses on tumour growth (Heise & Gorlich, 1966).

The mechanism by which androgens cause tumour regression is still unclear but the effect can be reversed by prolactin (Quadri, Kledzik & Meites, 1974). Although it has been demonstrated that prolactin receptors were reduced in tumours which regressed after testosterone treatment it was concluded that the extent of the receptor reduction was probably insufficient to account for androgen-induced mammary tumour regression (Costlow, Buschow & McGuire, 1976). It is also possible that androgens may interfere with oestrogen action at its receptor-binding level (Nicholson, Davies & Griffiths, 1978; Rochefort, Capony & Garcia, 1979). Specific cytoplasmic receptors for 5α-dihydrotestosterone have been identified in the DMBA-induced mammary tumour and may indicate a more direct action of androgens on tumour growth (Ip, Milholland, Kim & Rosen, 1978).

In addition to prolactin and oestradiol, insulin appears to be a hormone essential for the maintenance and growth of DMBA-induced tumours (Hilf, Hissin & Shafie, 1978). DMBA-induced tumours regress in animals rendered diabetic experimentally (Heuson & Legros, 1970 & 1972; Cohen & Hilf, 1974 & 1975), and will regrow upon the administration of insulin (Heuson, Legros & Heimann, 1972). Insulin receptors have been identified in the plasma membrane of DMBA tumour

cells (Hilf, Hissin & Shafie, 1978; Shafie & Hilf, 1978). The presence of insulin is also essential in cultured tumour system for maximal effects of pituitary and ovarian hormones to be demonstrated (Pasteels, Heuson, Heuson-Stiennon & Legros, 1976).

III. Steroid metabolism

Steroid metabolism can be considered in three parts.

(1) Synthesis is classically associated with the endocrine organs of the adrenal cortex, testis, ovary and placenta. In these organs cholesterol is formed from non-steroidal components and serves as precursor for the synthesis of steroid hormones. The pathway from cholesterol to form C-21, C-19 and C-18 steroids is common to all these tissues. Apart from adrenal cortex which is exclusive in its synthesis of corticosteroids these tissues differ from each other in their quantitative production of specific hormones. The steroids so produced are secreted into the circulation for transportation to target tissues where they can initiate biological responses by attachment to specific intracellular receptors.

(2) Target tissues have distinctive patterns of steroid metabolism. This can play a role in modulating responses of target tissues to steroid hormones by regulating the local levels of active steroid. The metabolism of steroids by target cells is not obligatory, however, and certain hormonal steroids are recovered unaltered after incubation with their target tissues (King & Mainwaring, 1974).

(3) The third area of steroid metabolism can be considered as deactivation and preparation for excretion by transformations such as hydroxylation and conjugation. The liver has a large capacity for such activity (Baird, Horton, Longcope & Tait, 1969).

In contrast to most other major classes of steroid which are secreted from endocrine organs in their ultimately active form, the C-19 steroids in the circulation, DHA, $DHASO_4$, 4-androstenedione and 5-androstenediol, which are precursors for the major androgen, testosterone, and testosterone itself, appear to require further conversion to 5α-dihydrotestosterone in situ for the expression

of most androgenic effects (Baulieu, Lasnitzki & Robel, 1968; Gloyna & Wilson, 1970).

Evidence that 5α-dihydrotestosterone functions as a proximal androgen has come from several sources. When tested in systems which assess androgenic potency, 5α-dihydrotestosterone was found to be highly active and usually the most potent of the naturally occurring steroids (Dorfman & Shipley, 1956; Lasnitzki, Whitaker & Withycombe, 1975). It has been demonstrated that 5α-dihydrotestosterone is formed from testosterone by male accessory organs (Pearlman & Pearlman, 1961; Farnsworth & Brown, 1963), and that it is bound by the nuclei of these organs (Anderson & Liao, 1968; Brucho vsky & Wilson, 1968; Tveter & Attramadal, 1968). The binding of 5α-dihydrotestosterone to cytoplasmic androgen receptors has been widely demonstrated and occurs more readily than that of testosterone (Liao, Liang, Fang, Castenada & Shao, 1973; King & Mainwaring, 1974). It has also been shown that 5α-dihydrotestosterone receptor complexes can stimulate DNA-dependent RNA polymerase activity in rat prostatic tissue (Davies & Griffiths, 1974).

The critical conversion of testosterone to 5α-dihydrotestosterone is irreversibly catalysed by the enzyme 5α-reductase (4-ene-3-ketosteroid, 5α-oxidoreductase). This enzyme has been found in both microsomal and nuclear fractions of the male accessory glands and appears to have an absolute requirement for NADPH (Brucho vsky & Wilson, 1968; Morfin, Alipoulios, Chamberlain & Ofner, 1970; Moore & Wilson, 1972).

In general the quantities of active steroid available for receptor binding are controlled by the modulatory action of hypophyseal trophic hormones on the metabolism and secretion of these steroids by endocrine glands. That the final step in the production of 5α-dihydrotestosterone occurs in the target tissues rather than in the endocrine glands provides another level for the control of

androgenic stimulation by modification of 5α-reductase activity in the target tissues.

Of particular relevance to the present work are reports of prolactin exerting an effect on 5α-reductase activity in adrenal cortex (Witorsch & Kitay, 1970 and 1972; Witorsch & Edwards, 1976), prostate (Helmerich & Altwein, 1976; Kurth, Jacobi, Sinterhauf & Altwein, 1977) and liver (Schriefers, Keck, Klein & Schroder, 1975; Skett, Eneroth & Gustafsson, 1978). Oestradiol has also been reported to modulate 5α-reductase in adrenal cortex (Kitay, Coyne & Swygert, 1970; Maynard & Cameron, 1973), prostate (Farnsworth, 1970; Griffiths, Harper, Groom, Pike, Fahmy & Pierrepoint, 1970; Belham & Neal, 1971; Leav, Morfin, Ofner, Cavazos & Leeds, 1971; Danutra, Harper & Griffiths, 1973; Fencl & Villee, 1973; Jenkins & McCaffery, 1974) and liver (Schriefers, 1967; Szamatowicz, 1974; Dieringer, Lamartiniere & Lucier, 1979). Although differences in protocol make comparisons between these reports difficult it appears that the hormones can induce either stimulatory or inhibitory effects on 5α-reductase dependent on the tissue under study. Most of these studies were based on the measurement of 5α-reductase activity after manipulations to raise or lower prolactin and oestradiol levels in plasma. Because of the complex hormonal interrelationships in both male and female endocrine systems it is possible that some of the observed changes in 5α-reductase activity could have resulted not only from direct actions of prolactin or oestradiol but from secondary changes in the plasma levels of other hormones.

Since breast cancer, in common with normal mammary gland, is a target for steroid hormones and possesses specific receptors for most categories of steroid it is not unexpected that it should metabolise steroids. Preliminary indications that breast cancer might be capable of steroid interconversion have been provided by observations of unusual patterns of steroid metabolites in the urine of breast cancer patients who had received ablative endodrine therapy (West, Damast, Sarro & Pearson, 1956; Bulbrook & Greenwood, 1957; Bulbrook,

Greenwood & Williams, 1960; Chang & Dao, 1961; Adams & Wong, 1968 b; Adams & Brown, 1971).

Early attempts to study the conversion of steroids by human breast cancer in vitro using non-labelled precursors revealed that human mammary gland, mammary fibroadenoma and adenocarcinoma all converted oestradiol to oestrone and that mammary cancer rapidly metabolised testosterone with 4-androstenedione as a major product (Ryan & Engel, 1953; Breuer, 1958; Wotiz, Lemon & Voulgaropoulos, 1954). In later studies the introduction of radioactive steroids as precursors permitted more accurate detection and identification of the metabolites formed during in vitro incubations.

Fig. 1 diagrammatically interprets the results of these studies with particular emphasis being given to the interconversions of C-19 steroids. The pathways boldly shown have been identified but may not have been invariably observed. The inability to confirm a conversion which others have shown to exist can result from the use of inappropriate incubation conditions, insensitive detection techniques, or the chance that some, but not all, tumours possess the necessary enzyme activity. The latter possibility has become apparent from the qualitative differences in metabolism observed in tumours of larger series incubated under identical conditions (de Thibault de Boesinghe & Lacroix, 1974; Miller & Forrest, 1976, Abul-Hajj, 1979).

Although cleavage of the side chain of cholesterol as an initial step in the synthesis of steroid hormones is usually considered an exclusive property of the classical endocrine organs, extensive metabolism of cholesterol by breast cancer has been demonstrated. It yields amongst its metabolites the C-21 steroids, progesterone and pregnenolone (Adams & Wong, 1969), and the C-19 steroids, testosterone, androsterone, 4-androstenedione, 5α-androstanedione and 5α-androstane-3α,17β-diol (Dao, Varela & Morreal,1972). Pregnenolone is converted to progesterone (Adams & Wong, 1968 a; Deshpande, Carson, Martino & Tarquini, 1976), and to 17α-hydroxyprogesterone, DHA and 4-androstenedione

(Abul-Hajj, Iverson & Kiang, 1979 c). Both α- and β-hydroxylation at the 20-position of 17α-hydroxyprogesterone and its conversion to 4-androstenedione have been demonstrated in human breast cancer (Adams & Wong, 1969).

The metabolism of DHA and $DHASO_4$, secreted in large quantities by the adrenal (Wieland, Levy, Katz & Hirschmann, 1963; Baulieu, Corpéchot, Dray, Emilozzi, Lebean, Mauvais-Jarvis & Robel, 1965) is of particular importance because of the high concentrations of $DHASO_4$ found in plasma (Yamaji & Ibayashi, 1969; Doouss, Skinner & Couch, 1975), and because of the possible implication of $DHASO_4$ in the aetiology of breast cancer in postmenopausal women (Adams, 1977). The presence of a sulphotransferase which controls the interconversion of DHA and its sulphate was demonstrated first in breast cancer by Adams in 1964 and has been subsequently confirmed by other workers (Dao & Libby, 1968; Jones, Cameron, Griffiths, Gleave & Forrest, 1970; Leung, Jack & Wood, 1972; Couch, Skinner, Tobler & Doouss, 1975). Incubation of DHA or $DHASO_4$ with normal or cancerous breast yields the oxidation product 4-androstenedione and the reduction product 5-androstenediol (Adams & Wong, 1968 a; Jones, Cameron, Griffiths, Gleave & Forrest, 1970; Couch, Skinner, Tobler & Doouss, 1975; Deshpande, Carson, Martino & Tarquini, 1976; Li, Foo & Adams, 1978). Both of these products could act as intermediates for the conversion of DHA to testosterone (Miller, McDonald, Forrest & Shivas, 1973; Miller, Forrest & Hamilton, 1974; Abul-Hajj, 1975). DHA is also converted to 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstanedione (Miller, Forrest & Hamilton, 1974; Abul-Hajj, 1975), presumably by the 5α-reduction of 4-ene products. 16α-Hydroxylase activity has also been noted in breast cancer tissue with the formation of 16α-hydroxyDHA, 5-androstene-3β,16α,17β-triol and 16α-hydroxytestosterone from DHA (Adams & Wong, 1968 a; Jones, Cameron, Griffiths, Gleave & Forrest, 1970; Miller, Forrest & Hamilton, 1974). Two recent reports describe extensive oxygenation of DHA at the 7-position by breast cancer and normal breast (Couch, Skinner, Tobler &

Doouss, 1975; Li, Foo & Adams, 1978). 7-Keto, 7α-hydroxy- and 7β-hydroxy-DHA are all formed from DHA and in fact the 7α-hydroxylated product appears to be the major metabolite of DHA in human breast cancer incubations. The physiological significance of the addition of ketone or hydroxyl groups to the 7-position of DHA is not yet clear. 5-Androstenediol also undergoes 7α-hydroxylation to form 5-androstene-3β,7α,17β-triol, a conversion which reduces the affinity of 5-androstenediol for oestrogen receptors (Li, Foo & Adams, 1978).

The main products formed during the incubation of normal and cancerous human breast preparations with testosterone are 4-androstenedione (Breuer, 1958; Jenkins & Ash, 1972), and the 5α-reduced products, 5α-dihydro-testosterone and 5α-androstanediol (Jones, Cameron, Griffiths, Gleave & Forrest, 1970; Miller, McDonald, Forrest & Shivas, 1973; Geier, Horn & Lichtshtein, 1974; Lippman, Bolan & Huff, 1976). 5α-Androstane-3α,17β-diol appears to be the major 5α-androstanediol formed (Li, Chandra, Foo, Adams & McDonald, 1976; Lloyd, 1979), although 5α-androstane-3β,17β-diol has also been identified as a metabolite (Griffiths, Jones, Cameron, Gleave & Forrest, 1972). In general the production of 4-androstenedione is slightly greater than that of the 5α-reduced products (Miller & Forrest, 1974 & 1976; Abul-Hajj, Iverson & Kiang, 1979 a). In addition to 5α-reductase, the presence of 5β-reductase has also been demonstrated in some but not all breast cancers (Abul-Hajj, 1979). The other C-19 metabolites which have been identified after the incubation of radioactively labelled testosterone with human breast cancer are 16α-hydroxytestosterone (Adams & Wong, 1968 a; Miller, Forrest & Hamilton, 1974), DHA (Miller, Forrest & Hamilton, 1974), 5α-androstanedione (Geier, Horn & Lichtshtein, 1974; Adams & Li, 1975), epitestosterone (Griffiths, Jones, Cameron, Gleave & Forrest, 1972) and androsterone (Geier, Horn & Lichtshtein, 1974).

Varela and Dao (1978) reported that 5α-androstanes form the main products of the metabolism of 4-androstenedione by human breast cancer. These products may be formed directly from 4-androstenedione or via its initial conversion to testosterone (Jones, Cameron, Griffiths, Gleave & Forrest, 1970; Varela & Dao, 1978). The oestrogen responsive MCF-7 cell line was capable of transforming 4-androstenedione to aetiocholanolone, whereas the oestrogen non-responsive cell line BT-20 was not (Levitz, Raju, Sklarew & Post, 1978).

Since the early tentative evidence for the formation of oestrogens by human breast cancers (Adams & Wong, 1968 a; Jones, Cameron, Griffiths, Gleave & Forrest, 1970; Dao, Varela & Morreal,1972), the conversion of both DHA and testosterone to oestradiol has been unequivocally demonstrated in homogenates of at least half of all breast cancers studied (Miller & Forrest, 1974 & 1976; de Thibault de Boesinghe, Lacroix, Eechaute & Leusen, 1974; Abul-Hajj, 1975; Adams & Li, 1975; Li, Chandra, Foo, Adams & McDonald, 1976; Abul-Hajj, Iverson & Kiang, 1979a) More recently Varela and Dao (1978) have shown that 4-androstenedione can also be aromatised by human breast cancer.

Their inability to demonstrate the production of oestrogens by breast cancer cells in long term tissue culture has led d'Agata and co-workers to conclude that the presence of fat or normal breast tissue may be responsible for the formation of oestrogens from C-19 steroids by breast cancer slices (d'Agata, Monaco, Lippman & Loriaux, 1978). However Abul-Hajj, Iverson and Kiang (1979 a) have recently shown that although human breast fat cells can aromatise C-19 steroids, oestradiol synthesis in fat occurs at a lower rate than in cancerous tumour. In agreement with Miller, Shivas and Forrest (1978), they were unable to demonstrate aromatisation by normal breast. The problem of the apparent lack of aromatisation by cultured human breast cancer cells has recently been resolved by MacIndoe (1979), who was able to demonstrate the

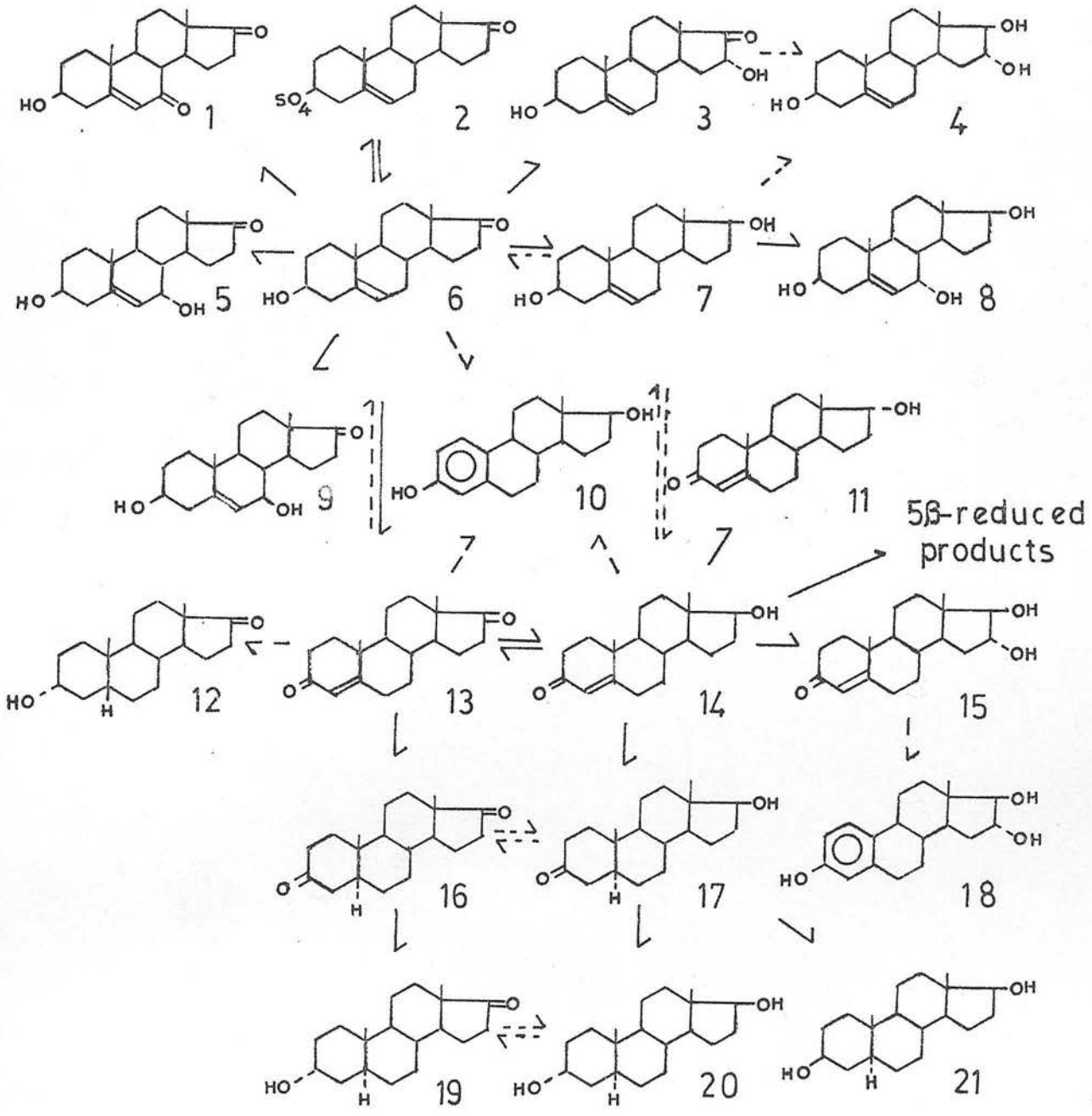
conversion of testosterone to oestradiol if serum was present in the incubation medium.

The recently demonstrated production of oestrone along with oestradiol from 4-androstenedione (Varela & Dao, 1978), and from DHA and testosterone (Abul-Hajj, Iverson & Kiang, 1979 a) agrees with earlier reports of a tumour 17β-hydroxysteroiddehydrogenase capable of converting oestradiol to oestrone (Ryan & Engel, 1953; Breuer, 1958; Melville, 1973; Geier, Horn, Levij, Lichtshtein & Finkelstein, 1975). In common with DHA, oestradiol is also sulphated by a sulphotransferase system and may also be converted to oestrone sulphate, 2-methoxyoestrone and possibly 2-methoxyoestrone sulphate in certain breast cancers (Melville, 1973). However, oestriol has not been found as a product of oestrogen metabolism in human breast cancer (Geier, Horn, Levij, Lichtshtein & Finkelstein, 1975; Abul-Hajj, Iverson & Kiang, 1979 b).

Steroid metabolism in the DMBA-induced rat mammary tumour has been less extensively investigated than human breast cancer. The possibility of the side chain cleavage of cholesterol or C-21 steroids does not appear to have been investigated in the DMBA-induced tumour although the formation of other C-21 steroids from progesterone has been recently reported (Mori, Tominaga & Tamaoki, 1978). Steroid metabolism studies using the DMBA-induced tumour model have been focussed on interconversions of C-19 steroids. The results of these studies are diagrammatically presented in Fig. 2.

The metabolism of DHA in DMBA-induced rat mammary carcinomas appears similar to that in human breast cancer. DHA is rapidly converted to 5-androstenediol (King, Panattoni, Gordon & Baker, 1965) and 7α-hydroxy-, 7β-hydroxy- and 7-ketoDHA (Li, Adams & Chandra, 1976) by DMBA-induced tumours and normal breast tissue. Testosterone, 5α-dihydrotestosterone and 5α-androstanediol have also been identified as metabolites of DHA in DMBA-induced tumours (Miller, Forrest & Hamilton, 1974). A sulphotransferase for DHA has also been observed in these tumours (Godefroi, Locke, Singh & Brooks,

Figure 1. Metabolism of C-19 steroids by human breast cancer



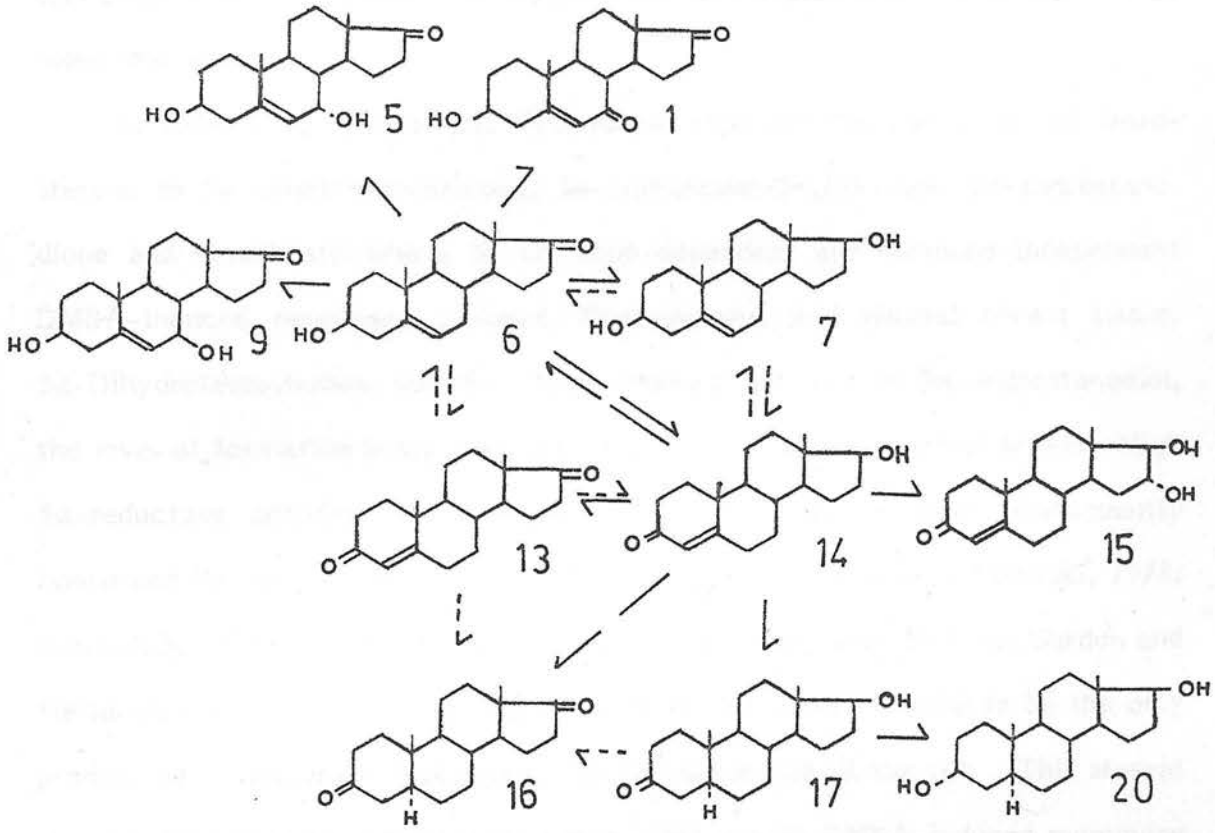
——→ confirmed conversions

— → confirmed conversions, but intermediates not shown

– – → possible conversions

See Figure 2 for key to steroids

Figure 2. Metabolism of C-19 steroids by DMBA-induced mammary tumours



⟶ confirmed conversions

⇢ confirmed conversions, but intermediates not shown

⇢ possible conversions

1. 7-keto DHA
2. DHA sulphate
3. 16α-hydroxy DHA
4. 16α-hydroxy 5-androstenediol
5. 7α-hydroxy DHA
6. DHA
7. 5-androstenediol
8. 7α-hydroxy 5-androstenediol
9. 7β-hydroxy DHA
10. oestradiol
11. epitestosterone
12. aetiocholanolone
13. 4-androstenedione
14. testosterone
15. 16α-hydroxytestosterone
16. 5α-androstanedione
17. 5α-dihydrotestosterone
18. oestriol
19. androsterone
20. 5α-androstanediol (3α)
21. 5α-androstanediol (3β)

1975). The importance of DHA metabolism in the rat may be less than in the human, as 4-ene- rather than 5-ene-C-19 steroids predominate in rat plasma (Dupon & Kim, 1973; Cutler, Glenn, Bush, Hodgen, Graham & Loriaux, 1978). In this respect the metabolism of testosterone and 4-androstenedione may be of more relevance.

In 1964 King, Gordon and Helfenstein reported the conversion of testosterone to 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol, 5α-androstanedione and 4-androstenedione by hormone-dependent and hormone-independent DMBA-induced mammary tumours, fibroadenoma and normal breast tissue. 5α-Dihydrotestosterone was the major product followed by 5α-androstanediol, the level of formation being greater in the tumours than in normal breast. High 5α-reductase activity in DMBA-induced carcinomas has been subsequently confirmed (Miller, Forrest & Hamilton, 1974; Mori, Tominaga & Tamaoki, 1978; Abul-Hajj, 1979). However, in contrast to the earlier work of King, Gordon and Helfenstein (1964), Mori's group found androst-4-ene-3α,17β-diol to be the only product of testosterone metabolism by normal breast of the rat. This steroid was not formed when testosterone was incubated with DMBA-induced mammary tumours (Mori, Tominaga & Tamaoki, 1978). Testosterone is also converted in small amounts to DHA and 16-OH testosterone (Miller, Forrest & Hamilton, 1974), but no evidence has been found for the 5β-reduction of testosterone (King, Gordon & Helfenstein, 1964; Abul-Hajj, 1979).

In contrast to human breast cancer, the presence of an aromatase system in DMBA- induced mammary cancer has not been conclusively demonstrated. Oestradiol in very low amounts was tentatively identified as a metabolite of both DHA and testosterone in two DMBA-induced tumours from the same rat (Miller, Forrest & Hamilton, 1974). However this result has not yet been confirmed and other tumours have provided negative results (King, Gordon & Helfenstein, 1964; King, Panattoni, Gordon & Baker, 1965; Miller, Forrest & Hamilton, 1974).

The DMBA-induced carcinoma of the rat also differs from human breast cancer in that the 5α-reduced products of testosterone account for a far greater percentage of the metabolism in the rat model than does 4-androstenedione.

From the limited work on oestrogen metabolism in the rat tumour model it appears that oestrone is converted to oestradiol (King, Panattoni, Gordon & Baker, 1965), and oestradiol is converted to water soluble products (Jellinck, Smith & Cleveland, 1975).

In addition to the human and rat, steroid metabolism also appears to be a feature of canine mammary cancer (Evans, Harvey, John & Pierrepoint, 1977), and of mammary cancer in mice whether arising spontaneously (Smith & King, 1966), or maintained as specialised androgen-dependent and androgen-independent tumour lines (Bruchovsky & Meakin, 1973; Gordon, Smith & King, 1974; Yamaguchi, Kasai, Minesita, Kotoh & Matsumoto, 1974).

Since steroid metabolism would appear to be a common feature of all breast cancers it is important to understand its role in the disease. The high capacity of the DMBA-induced rat mammary tumour to form the active androgen, 5α-dihydrotestosterone, and the ability of human breast cancer to form both 5α-dihydrotestosterone and oestradiol from precursor steroids available from the circulation may be relevant to tumour growth. However, the physiological significance of these conversions in breast cancer is not known.

With regard to the relationships between steroid metabolism and other parameters, King, Panattoni, Gordon & Baker (1965) reported that the conversion of oestrone to oestradiol correlated positively with the growth rate of hormone-dependent DMBA-induced tumours but that there was no relationship between 5α-reductase and tumour growth in the same tumours. Hormone dependency was assumed if tumours regressed after ovariectomy and regrew after the administration of oestradiol plus progesterone but it was not stated whether tumour growth and steroid metabolism were measured before or after endocrine manipulation. Although Mori, Tominaga and Tamaoki (1978) noted that the most

poorly differentiated tumour of a small series of DMBA-induced rat mammary tumours showed the highest 5α-reductase activity, a relationship between tumour histology and 5α-reductase activity has not been established. Using testosterone as a precursor Abul-Hajj (1979) found that, although 5α-reductase activity correlated well with oestrogen receptor content in human breast cancers, there was no such relationship in DMBA-induced rat mammary tumours. However the range of values obtained for both parameters in the rat tumours was much narrower than in the human tumours.

The relationships outlined above have been derived almost exclusively from the spontaneously arising variations between tumours with respect to steroid metabolism and the other parameters selected for study. No attempt has been made to alter these parameters, including that of steroid metabolism. In fact, very little is known about the control of the activity of enzymes which metabolise steroids in breast cancer.

Recent evidence has indicated, however, that androgen metabolism in DMBA-induced rat mammary tumours might be influenced by hormones. Tumours induced in rats rendered hyperprolactinaemic by chronic perphenazine administration from 30 days of age had a greater capacity to form both 5α-dihydrotestosterone and 5α-androstanediol from testosterone than had tumours from normoprolactinaemic rats (Miller, Buchan & Forrest, 1974; Miller, 1974 & 1976 a). Since perphenazine was administered both prior to and after tumour induction (50 days of age), it is not possible to determine whether the changes occurred as a result of the induction of tumours with a different potential for testosterone metabolism or whether alterations occurred during tumour growth. It has also been reported that oestradiol is capable of lowering 5α-reduction of testosterone by DMBA-induced mammary tumours both in vitro and in vivo (Miller, 1976 b & c).

The intention of the present study was to deliberately alter the hormonal status of rats bearing DMBA-induced mammary adenocarcinomas in a manner

known to affect the growth of these tumours. Any resultant changes in steroid metabolism of the tumours would not only provide the opportunity to study possible relationships between steroid metabolism and other tumour parameters, but also provide an insight into the control of the activity of steroid metabolising enzymes in this tumour.

Since, as discussed earlier, prolactin and oestradiol can profoundly influence the growth of DMBA-induced mammary tumours plasma levels of these two hormones were specifically altered and closely monitored. Attention was focussed on the ability of the tumour to metabolise testosterone, in particular by 5α-reduction, for the following reasons:- DMBA-induced tumours regress on androgen therapy; 5α-reductase is associated in many tissues with the expression of androgenic effects; 5α-reductase activity is high in DMBA-induced tumours and appears to constitute the main route of metabolism of testosterone.

Some of the results obtained in the course of this study have been presented elsewhere (Buchan, Fraser & Miller, 1976; Buchan & Miller, 1978 & 1980).

METHODS

I. Chemicals and reagents

With the exception of those listed below, all chemicals were obtained from BDH Chemicals Ltd, Poole, or Fisons Scientific Apparatus, Loughborough. These were of analar grade and stored according to the suppliers' specifications. Ethanol, also of analar grade, was redistilled twice before use. Tap water was deionised and distilled before use.

Reagent	Source
Calf thymus DNA, type I : sodium salt	Sigma, London
7, 12-Dimethylbenz [a] anthracene	Sigma, London
Glucose-6-phosphate	Sigma, London
Glucose-6-phosphate dehydrogenase from torula yeast, type XI, (EC No 1.1.1.49)	Sigma, London
Nicotinamide adenine dinucleotide phosphate	Sigma, London
Norit A charcoal	Sigma, London
Anti-oestradiol-17β serum (rabbit 348/bleed 6)	Dr W Hunter, MRC Reproductive Biology Unit, Edinburgh
CB 154	Sandoz, Basle, Switzerland
Corn oil (commercial sample)	Boots, Nottingham
Cotton seed oil (commercial sample)	Valvona & Crolla, Edinburgh
Dextran T-70	Pharmacia, Uppsala, Sweden
Sephadex LH-20	Pharmacia, Uppsala, Sweden
Donkey anti-rabbit serum RD-17	Burroughs Wellcome, Beckenham
Horse serum	Burroughs Wellcome, Beckenham
Hexamethyldisilazane	Koch-Light, Colnbrook
Histological stains	R A Lamb, London

Fentazin injection (1ml ampoules)	Allen & Hanburys, London
Perphenazine BP	Allen & Hanburys, London
Heparin	Weddel Pharmaceuticals, London
Kieselgel $HF_{254 + 366}$ (Typ 60)	Merck, Darmstadt, W Germany
Liquifluor	NEN Chemicals, Dreieich, W Germany
3% OV-1 on gas chrom Q	Applied Science Labs, Philadelphia, USA
Rat prolactin RIA kit	NIAMDD, Bethesda, USA

Non-radioactive steroids were supplied by Steraloids, Croydon; Sigma, London; or Koch-Light, Colnbrook. 7α-hydroxytestosterone was supplied by Searle, Chicago, USA, and androst-4-ene-3α, 17β-diol was a gift from Schering, Berlin, W Germany. All steroids were stored at 4°C.

[7α- ^{3}H] Testosterone, [4 - ^{3}H] toluene, and $Na^{125}I$ were obtained from the Radiochemical Centre, Amersham. [2, 4, 6, 7 - ^{3}H] Oestradiol was obtained from NEN Chemicals, Dreieich, W Germany.

II. Purification of steroids

[^{3}H] Testosterone was found to be chromatographically homogeneous in the major solvent systems used and had the same chromatographic mobility as authentic unlabelled testosterone. Nevertheless the precursor was purified immediately before use by t.l.c. in solvent system III.

[^{3}H] Oestradiol was purified by a method based on that of Mikhail, Wu, Ferin and Van de Wiele (1970). 20-40 µCi [^{3}H] oestradiol in benzene : methanol (85:15 v/v) was applied to a column of Sephadex LH-20 (175mm x 10mm) and eluted with the same solvent. Radioactivity was measured in 20µl portions of 1ml fractions of eluate and only the 2-3 peak fractions were pooled to yield purified [^{3}H] oestradiol.

III. Animal procedures

1. Animals

Female albino rats of the Sprague-Dawley strain were used throughout. These animals came from a random-bred colony, maintained in the Department of Clinical Surgery, whose original breeding stock were obtained from Oxford Laboratory Animal Colonies (Bicester, Oxfordshire) in 1973. After weaning at 21 - 26 days of age, females were isolated from males and kept virgin. Caged in groups of 2 - 5 these animals were housed at $21\pm2^{o}C$ with daily lighting from 0700 - 1900 hours. Diet consisted of standard rat cake (MacGregor & Co (Leith) Ltd, Quayside Mills, Leith, Scotland) and tap water was provided ad libitum.

2. Tumour induction

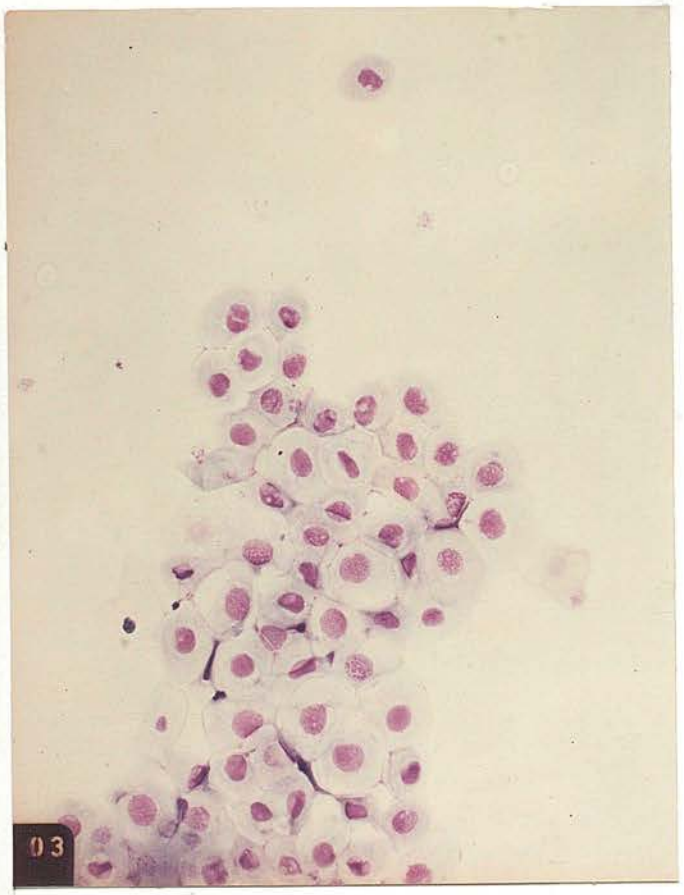
At 47 - 55 days of age, a sample of blood was taken from a tail vein of each rat for assay of plasma prolactin and the stage of oestrous cycle assessed by vaginal smear. A single dose of 30mg DMBA dissolved in 2ml cotton-seed oil was then administered by intragastric instillation. These procedures were carried out between 1400 and 1600 hours under light ether anaesthesia.

The animals, individually identified by ear markings made at DMBA administration, were palpated weekly for mammary tumours. Once palpable, two perpendicular diameters of the tumours were measured two or three times weekly by caliper in the conscious animal. Tumour size was defined as the product of the two diameters and expressed in cm^2. Animals bearing no actively growing tumours at 120 days after receiving DMBA were not used for tumour studies.

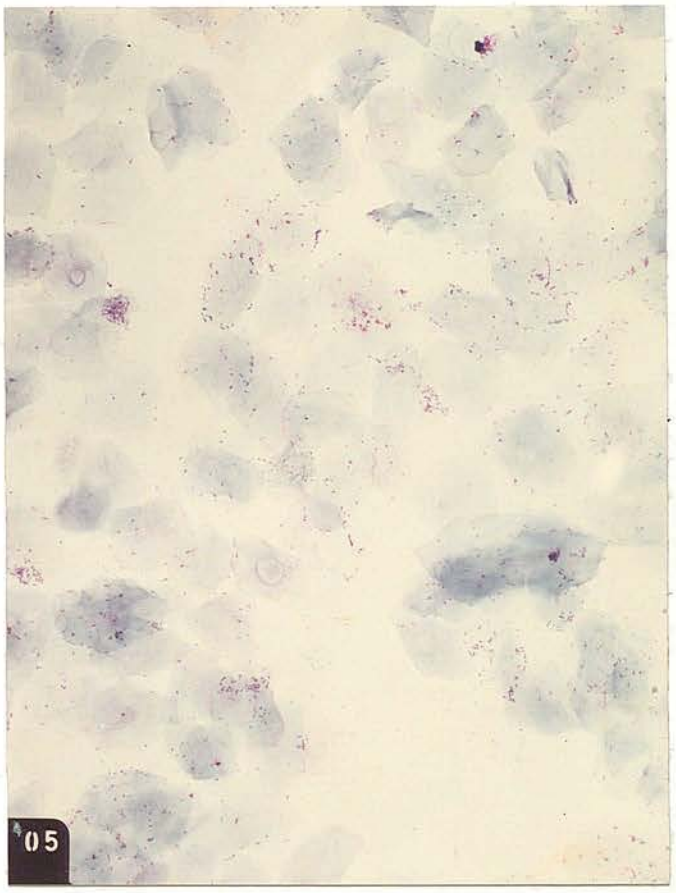
3. Vaginal smears

Daily vaginal smears were taken from rats with actively growing tumours above about $1.5cm^2$ in size and from rats used for plasma hormone studies to monitor the stage of the oestrous cycle. Smears were obtained by vaginal lavage and after microscopic examination classified according to the description

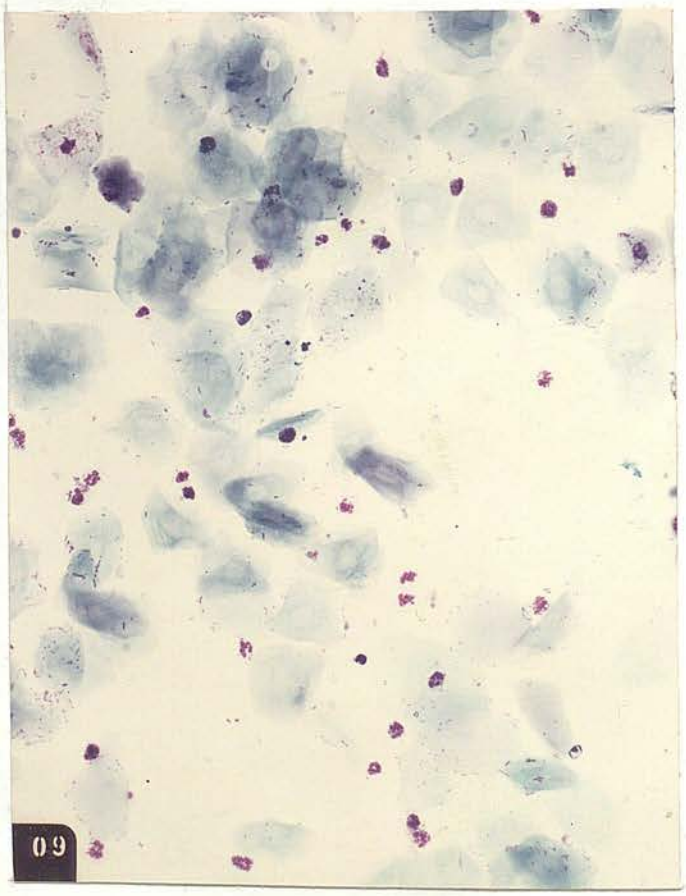
Figure 3. Cytology of vaginal smears in the cycling rat



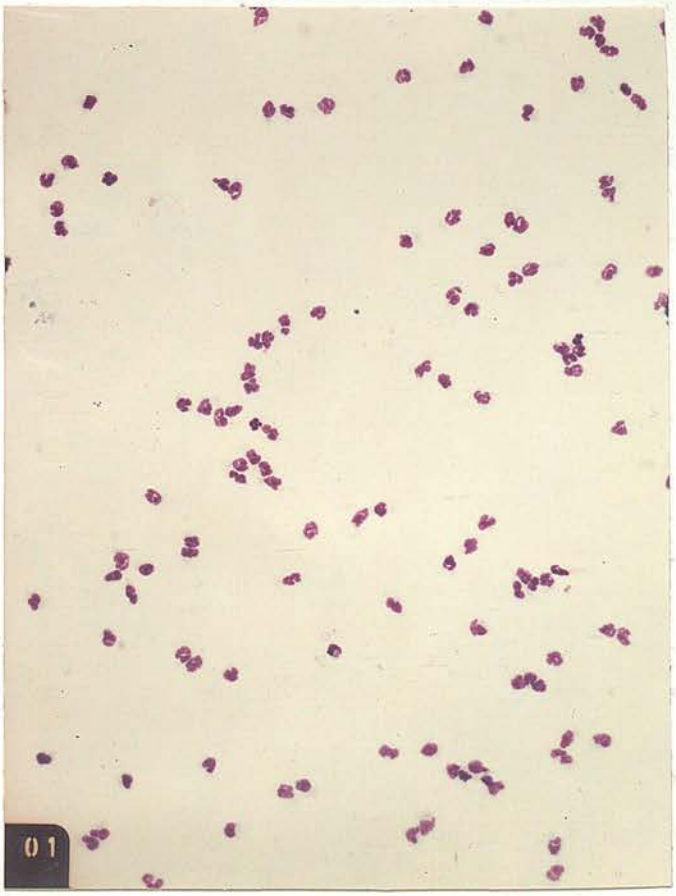
Proestrus



Oestrus



Metoestrus



Dioestrus

of Long & Evans (1922). Photographs of the cell types seen in vaginal smears taken at different stages of the oestrous cycle are shown in Fig. 3. Cells were stained by the May-Grunwald-Geimsa technique (Baker, Silverton & Luckcock, 1966) and are magnified 250-fold.

4. Anaesthesia

All operative procedures and the removal of blood samples were conducted under ether anaesthesia. Anaesthesia was induced by placing the animal in a large airtight jar containing an atmosphere saturated with ether. For longer operative procedures anaesthesia was maintained by inhalation of ether from a swab in a small nose-cone.

5. Blood collection

Two methods were employed for the collection of blood for assaying plasma hormone levels. The method which could be used repeatedly in individual animals was to drain a volume of approximately 1ml into a heparinised glass tube from a small incision of a tail vein. Since these samples were taken mainly for the estimation of plasma prolactin, a hormone whose levels can alter dramatically with stress (Nicoll, Talwalker & Meites, 1960; Stern & Voogt, 1973), every attempt was made to obtain the sample as soon as possible after the animal was removed from its normal environment (usually within two minutes of removal from animal house). The second method of blood collection was performed at sacrifice and involved exsanguination via the abdominal aorta into a heparinised syringe. Blood was immediately transferred to a heparin-coated plastic tube for plasma separation.

Both tail vein and aorta blood samples were put on ice and then centrifuged at 2,500 r.p.m. for 10 minutes at $4^{o}C$ using a Mistral 6L centrifuge (MSE Ltd, London). The resultant plasmas were stored at $-20^{o}C$ either in 100µl aliquots in polythene tubes for prolactin assay or in bulk in acid-washed glass vials for oestradiol assay.

6. Surgical procedures

Rats were bilaterally ovariectomised through two dorsal skin incisions. The ovary and a distal piece of the uterus were ligated, severed and removed, and the incisions sutured.

7. Drugs and steroid treatment

All drugs and steroids were dissolved or finely suspended in corn oil and administered daily by subcutaneous injection into the inguinal region. The dilution in corn oil was such that the required daily dose was always achieved by injecting 1ml of the solution/kg body weight: for example, if the required dose was 5mg/kg a 200g rat would receive 0.2ml of a 5mg/ml solution. When appropriate, control animals received the relevant volume of corn oil.

8. Treatment protocol

The central part of the work involved assessing the effects of five different hormonal manipulations on certain tumour parameters. The five hormonal manipulations which were each applied to groups of rats were (a) intact control, (b) intact + perphenazine, (c) intact + CB 154, (d) ovariectomised control, (e) ovariectomised + perphenazine. The animals selected for these groups were required to have at least one actively growing tumour greater than $2.0cm^2$ in size and to be displaying regular 4-day cycles. Treatment was initiated on the day of dioestrus and normally lasted 12 days so that animals which continued to cycle were sacrificed on the day of dioestrus. In these groups of animals plasma was collected from the tail vein immediately prior to the start of treatment and on the day of sacrifice for plasma prolactin assay. Also at sacrifice blood was collected from the aorta for oestradiol assay.

9. Tumours

At sacrifice tumours were immediately excised and processed on ice. Portions were taken for histology, steroid metabolism studies and, when suff-

icient material was available, DNA estimation and measurement of oestradiol receptor activity. Necrotic tumours were discarded.

The background to other animals which were not in treatment groups listed above, but whose tumours were used for steroid metabolism studies, are described in the relevant parts of the RESULTS section.

IV. Tumour histology

Representative portions of tumour were fixed in 10% formal saline for 24 hours, automatically processed, sectioned and stained in Mayer's haematoxylin and eosin (Baker, Silverton & Luckcock, 1966).

V. DNA extraction and estimation

The method was a modification of that of Burton (1956). Weighed duplicate portions of tumour (25 - 40mg) were finely cut and homogenised by hand with a ground glass mortar and pestle in 5ml distilled water. This was transferred to a centrifuge tube washing the homogeniser with 2 x 2.5ml 10% (w/v) trichloroacetic acid which was bulked with the homogenate. The precipitate was then allowed to settle for at least 15 minutes at room temperature before centrifugation at approximately 2,000 r.p.m. on a bench centrifuge (MSE Ltd, London) for 3 minutes. The supernatant was discarded and the pellet twice resuspended in 5ml of 5% (w/v) trichloroacetic acid, centrifuged and the supernatants discarded. The precipitate was then suspended in 0.5M perchloric acid (4.0ml), heated at 70°C for 15 minutes, with occasional mixing, and centrifuged. The supernatant was carefully decanted off and the precipitate resuspended in a further 2.0ml of 0.5M perchloric acid and heated and centrifuged as before. The supernatant was bulked with the previous extract and mixed. Duplicate aliquots (2ml) were then estimated colourimetrically (600nm) after incubating overnight at 30°C with diphenylamine/acetaldehyde

reagent (Burton, 1956). The results were read off a linear calibration curve constructed with calf thymus DNA standards.

VI. Oestrogen receptor assay

The method used was based on saturation analysis as described by Hawkins, Hill and Freedman (1975), with slight modifications (Hawkins, Hill, Freedman, Killen, Buchan, Miller & Forrest, 1977). In brief, tumours were left in tris buffer solution (0.25M sucrose, 10mM tris (hydroxymethyl) aminomethane and 1mM ethylene diamine tetra-acetate pH 8.0), containing 0.5mM dithiothreitol, for one hour prior to homogenisation (15 seconds disruption + 45 seconds rest x 3 using the Silverson mixer emulsifier) in tris buffer (300mg/ml). The homogenate was centrifuged at 105,000g for 50 minutes and the resultant supernatant used for assay of the cytoplasmic receptors. Duplicate tubes containing 10pg [^{3}H]-oestradiol (100µl tris), 0, 10, 30, 50, 70, 90 or 20,000pg non-radioactive oestradiol (1ml tris) and 100µl tumour supernatant were allowed to equilibriate overnight. Unbound steroid was separated by adding 0.5ml of a dextran-coated-charcoal solution (1.5g charcoal, 0.015g dextran/1 tris buffer) to the tubes which then stood for 15 minutes before centrifuging for 10 minutes at 2,500 r.p.m. The supernatant (bound) fraction was decanted directly into a counting vial containing 5ml organic scintillator solution (21ml Liquifluor, 10ml ethanol and 49ml toluene). All procedures were conducted at 4^{o}C. Two 100µl aliquots of the [^{3}H]-oestradiol solution plus an appropriate amount of buffer were added directly to the scintillant to determine total counts added. "No protein" and "no charcoal" blanks were also included to determine the efficiency of the charcoal suspension and the decanting step respectively. After correcting for non-specific binding by subtraction of the mean value for 20,000pg the ratio of radioactivity bound over radioactivity free on the ordinates is plotted against the mass of total oestradiol bound (pg) on the abscissa according to Scatchard (1949). The concentration of oestrogen receptor

binding sites (in f mol/g tissue) and dissociation constant were then calculated from intercept on the ordinate and gradient of the line respectively.

VII. Incubation of tumour homogenates for steroid metabolism

All chemicals and solutions were stored at $4^{o}C$ and all procedures prior to the start of the incubation were carried out on ice. A portion of tumour (wet weight = 0.500g) was finely sliced and sonicated twice using an Ultrasonic A180G sonicator (Shipley, Yorkshire), adjusted to maximum noise at power position 8 for 1 minute with a 1 minute interval in 5ml Krebs-Ringer solution buffered with phosphate (Krebs & Henseleit, 1932). The final concentrations of the components in this medium were NaCl, 118mM; KCl, 4.7mM; $CaCl_2$, 2.5mM; $K_2H\ PO_4$, 1.18mM; $MgSO_4$, $7H_20$, 1.18mM; phosphate buffer, 16mM. The stock solution of phosphate buffer consisted of 17.8g $Na_2HPO_4\ .2H_20$ plus 20ml HCl, 1M, made up to a volume of 1 litre with distilled water. The pH of the final solution was adjusted to 7.4 at room temperature by the addition of NaOH. The homogenate was transferred to a conical flask containing an NADPH-generating system of 100 umol glucose-6-phosphate, 15 µmol $NADP^+$ and 25 units glucose-6-phosphate dehydrogenase (EC 1.1.1.49). A small volume of Krebs buffer was added to the solution of glucose-6-phosphate dehydrogenase supplied by Sigma to permit the accurate addition of 25 units in a convenient volume (25 or 100µl).

To the final volume of 7.5ml was added 50 x 10^6 d.p.m. ($\simeq$ 750ng) of freshly purified [7α-^3H] testosterone in 50µl of a solution of propylene glycol : ethanol (1:1, v/v). Incubation systems were aerated with a mixture of 95% 0_2/5% CO_2, stoppered and incubated by shaking at $37^{o}C$ for one hour in a water bath (Gallenkamp, London).

The reaction was stopped by the addition of approximately 30ml methanol to the flask which was stored at $-20^{o}C$ until the extraction and identification of steroids.

VIII. Extraction of steroids

Before extraction of steroids from the incubation mixture 500ug of non-radioactive carrier steroid in 0.5ml ethanol were added for the precursor and for each metabolite to be investigated. The carrier steroid served to monitor recovery losses during subsequent procedures and to identify the steroid on t.l.c. plates on examination under u.v. light. Aliquots of carrier were also used to prepare u.v. spectrophotometric and g.l.c. standards.

The mixture was then centrifuged at approximately 2,500 r.p.m. on a bench centrifuge (MSE Ltd, London) for two minutes and the supernatant decanted off. The pellet was homogenised twice in 20ml acetone washings of the incubation flask using a Silverson mixer emulsifier (Silverson Machines Ltd, London), (one minute at maximum r.p.m.) and the acetone supernatants bulked with the aqueous methanol supernatant. Aliquots were taken for estimation of total initial radioactivity. The supernatants were evaporated to dryness under a partial vacuum at 50°C using a rotary evaporator (Buchi, Switzerland). The residue was dissolved in 20ml ethyl acetate and partitioned with 20ml distilled water. The ethyl acetate phase was separated off and the aqueous fraction extracted with a further 20ml ethyl acetate. The pooled ethyl acetate fractions were evaporated to dryness and redissolved in 0.2ml ethanol for initial separation of steroids by t.l.c. The aqueous fraction was retained until t.l.c. of the organic fraction.

IX. Thin layer chromatography

1. Preparation of plates

A slurry of Kieselgel $HF_{254 + 366}$ (30g in 72ml distilled water) was spread over glass plates (20cm x 20cm) using a t.l.c. spreader (Shandon Scientific Company Ltd, London), set for a depth of 0.25mm. Plates were then dried at 100°C for two hours and kept desiccated until used.

2. Solvent systems

The solvent systems used are listed in Table 1. The choice of solvent system used for particular steroids is indicated in Table 2. The Roman numerals following the reactions listed in this table refer to the solvent system used to separate the products of those particular reactions.

3. Detection of steroids

The unlabelled carrier steroids and their derivatives were visualised under a Camag TL-900/U Universal u.v. lamp (Camlab, Cambridge) with two wavelength settings. At the 254nm wavelength emission 4-ene, 3-ketosteroids appeared as a deep purple colour on a neutral background, whereas at 350nm 3-hydroxylated and 5α-reduced steroids and their derivatives appeared as a pale white or yellow area against a blue background. For comparison authentic standards were run in channels of the t.l.c. plate adjacent to those carrying the samples. In addition the distribution of radioactivity on the plates was located using a Panax RTLS-1A scanner with SREC-2 chart recorder (Panax Equipment Ltd, Surrey).

4. Elution of steroids

After detection the area of silica gel containing the steroid was scraped from the plate and eluted by one of two methods. In the first method, employed in the preliminary stages of separation, silica gel was scraped into a micro-column stoppered with a glass wool filter. The silica gel was deactivated with a few drops of water and steroids eluted from the gel into a test tube with 10ml of ethanol. Prior to determination of specific activities, a second method of elution was adopted in which the silica gel was scraped into a test tube, a few drops of water and 5ml of ethanol added and the contents mixed. After centrifugation for two minutes at approximately 2,000 r.p.m. the eluate was decanted off. The silica was resuspended in two further rinses of ethanol (2.5ml each) and after centrifugation these eluates were bulked with the previous eluate. Eluates from both procedures were dried in a stream of air at 60^{o}C.

Table 1

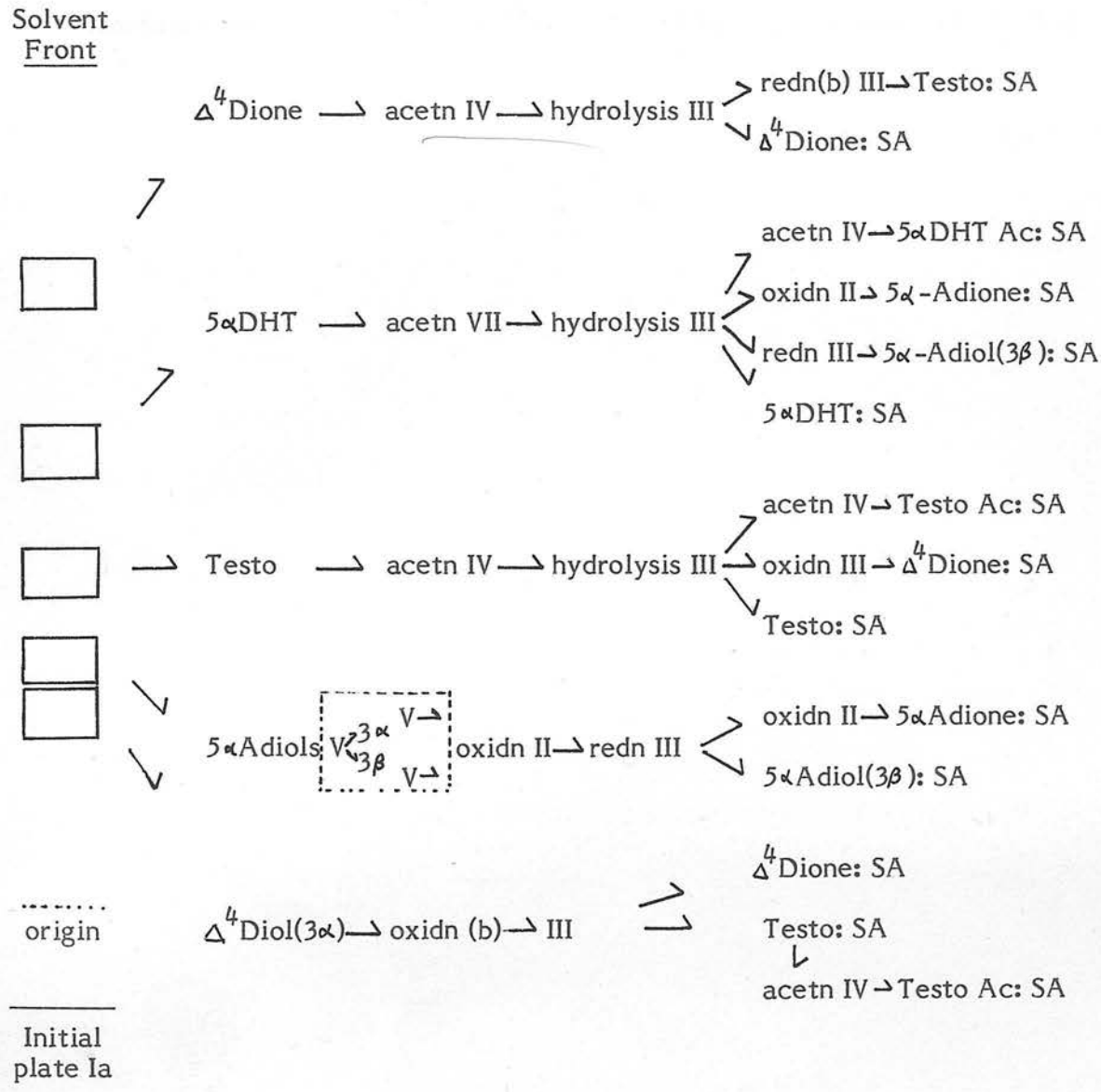
Solvent systems for thin layer chromatography

SYSTEM	SOLVENTS (RATIO OF VOLUMES)		TIME (Hours)	CONDITIONS
Ia	Chloroform : acetone	(98:2)	$2\frac{1}{2}$	continuous elution tank
Ib	Chloroform : acetone	(185:15)	2	continuous elution tank
II	Chloroform : acetone	(98.2)	1	Sealed tank
III	Chloroform : acetone	(185:15)	1	"
IV	Cyclohexane : ethyl acetate	(70:30)	1	"
V	Diethylether : ethyl acetate	(95:5)	1	"
VI	Benzene : ethanol	(90:10)	1	"
VII	Cyclohexane : chloroform : ether : acetone	(140:10:50:1)	4 x 1	"

Silica gel $HF_{254 + 366}$ plates were used in all systems. The solvent in the sealed Shandon Chromatank was run to the top of the plate (running time = approximately one hour). In system VII plates were dried between each run. The selection of these systems is indicated in Table 2.

Table 2

Characterisation of metabolites of testosterone



Roman numerals refer to the solvent systems listed in Table 1.

Δ^4Dione = 4-androstenedione; Δ^4Diol(3α) = 4-androstene-3α, 17β -diol;

Testo = testosterone; 5αAdiol(3α) = 5α-androstane-3α, 17β -diol;

5αAdione = 5α-androstanedione; 5αAdiol(3β) = 5α-androstane-3β, 17β -diol;

5αDHT = 5α-dihydrotestosterone; 5αAdiols = 5αAdiol (3α) + 5 Adiol(3β);

Ac = acetate; acetn = acetylation; oxidn = oxidation; redn = reduction;

SA = specific activity determined.

(b) indicates the use of alternative methods of oxidation or reduction (M-14)

[ ] = steps omitted in determination of total 5αAdiols.

X. Formation of steroid derivatives

1. Acetylation

Acetates were prepared by the method of Zaffaroni and Burton (1951). The dried steroid was mixed with six drops acetic anhydride and three drops pyridine and incubated for one hour at 60°C or overnight at room temperature. 1ml of methanol was added at the end of the incubation to facilitate the drying of the reaction products at 60°C under a gentle air flow.

2. Hydrolysis of acetates

Steroid acetates were hydrolysed by overnight incubation at 37°C in 1ml methanol + 0.25ml 2% K_2CO_3 in distilled water.

3. Reduction

(a) Apart from 4-androstenedione, reduction was performed by adding 10mg solid $NaBH_4$ to the steroid dissolved in 1ml methanol and leaving the mixture for 15 minutes at room temperature.

(b) 4-androstenedione was reduced to testosterone by incubation for 35 minutes at 0°C with 0.1ml of freshly prepared 0.1% $NaBH_4$ in methanol.

4. Oxidation

(a) Apart from 4-androstenediol (3α) oxidation of steroids was performed by the method of Griffiths, Grant and Whyte (1963). Steroids were incubated at room temperature for 20 minutes in 0.5ml of a freshly prepared solution of 5ml acetone + 50µl of a stock oxidising solution (1.61g sodium dichromate/litre 5.2N H_2SO_4).

(b) 4-androstenediol (3α) was oxidised to testosterone by adding 100mg of manganese dioxide to the steroid in 0.5ml chloroform and the mixture shaken overnight at 37°C. This preparation was adapted from the method of Attenburrow, Cameron, Chapman, Evans, Hems, Jansen and Walker (1952).

Apart from the acetates all steroids were recovered by extracting with 2 x 5ml ethyl acetate after the addition of 3ml distilled water.

XI. Determination of non-radioactive steroids

1. Spectrophotometry

Testosterone, testosterone acetate and 4-androstenedione were dissolved in ethanol and absorption peak height measured at 240nm against ethanol in the reference cell using an SP800 spectrophotometer (Pye Unicam, Cambridge). The steroid content was calculated by comparison with standard solutions after correction against a silica gel blank taken through the same procedure as the steroid.

2. Gas-liquid chromatography

All other steroids were measured by g.l.c. analysis over a 5 ft glass column (diameter = 4mm) containing pretested 3% OV-1 coating on Gas Chrom Q (100 - 120 mesh). The column was silanised by overnight steeping in a solution of 5% hexamethyldisilazane in toluene (5 w/v). The column was packed under reduced pressure and conditioned overnight (16 hours) at 230°C with a carrier gas flow rate of 60ml/minute. Chromatography was conducted at 220°C with a carrier gas (argon) flow rate of 60ml/minute in a Pye 104 Chromatograph with Flame Ionisation Detector, model 2a, (Pye Unicam, Cambridge).

Internal standards, testosterone for free steroids and testosterone acetate for acetylated steroids, were added in the same quantity to all samples and standards. In each batch there were included a minimum of three standards to span the range of quantities in the samples. From the ratio of sample peak height to internal standard peak height and the mean of comparative values for consistent standards (less than 10% variation) the quantity of steroid in the sample was determined.

XII. Measurement of radioactivity

Aliquots from steroid solutions were dried in glass scintillation vials (FGB-Trident, Avon) and dissolved in 10ml of a mixture of 42 parts "liquifluor", 1,000 parts toluene. Radioactivity was measured with a Packard Tricarb Liquid Scintillation Counter (Packard Instrument Company Inc, Illinois, USA). Counting efficiency was routinely checked using a tritiated toluene standard of specified radioactivity and decay rate supplied by the Radiochemical Centre, Amersham. The radioactivity of each sample was determined by correcting the measured count rate for background and counting efficiency.

XIII. Expression of steroid metabolism results

Following purification by derivative formation and chromatography the specific activities (d.p.m./nmol) were calculated for steroids and their derivatives. Once the specific activities between the parent steroid and its derivatives were constant (all within 5% of the mean) the mean value was used to calculate the formation of each characterised steroid as a percentage of the total radioactivity using the recovery of cold steroid to correct for losses.

XIV. Plasma prolactin assay

The plasma prolactin concentration was determined by radioimmunoassay using a kit provided by NIAMD (Hawkins, Freedman, Marshall & Killen, 1975). Each plasma sample was assayed in duplicate (2 x 100µl) along with standard solutions of rat prolactin (NIAMD RP-1, $\frac{25}{32}$ to 400ng/ml in doubling dilutions). Horse serum (100µl), which contains negligible rat prolactin-like activity, was added to standard curve tubes so that their protein content was approximately similar to that of the plasma samples. Radioligand solution (100µl) containing 20,000 c.p.m. ^{125}I-prolactin prepared from purified rat prolactin (NIAMD-RP-I-2) and Na ^{125}I by oxidation by chloramine-T (Greenwood, Hunter & Glover,

1963) was mixed with the non-radioactive prolactin samples before the addition of 200µl rabbit anti-prolactin antibody (NIAMD anti-RP-serum 2) at a dilution of 1:25,000. After a three-day equilibration period at 4°C separation of free and bound ligand was achieved by a further incubation for one day at 4°C with donkey anti-rabbit serum (Burroughs Wellcome RD-17, diluted 1:6; 200µl per tube) followed by centrifugation at 3,000 r.p.m. for 30 minutes at 4°C in a Mistral 6L centrifuge (MSE Ltd, London). The radioactivity in the precipitate was determined at approximately 60% efficiency in a Wallac gamma sample counter GTL 300-500 (Wallac, Turku, Finland). A value for the concentration of prolactin in an unknown sample was read from a plot of c.p.m. bound vs $\log_{10}$ prolactin concentration for the standard tubes. Because the reproducibility of the assay varied due to the inevitable deterioration on storing certain reagents, a correction was made in each assay with data from quality controls of rat plasma stored under the same conditions as unknown samples. (see Appendix III)

XV. Plasma oestradiol assay

Plasma oestradiol-17β concentration was determined by radioimmunoassay using modification of the method of de Jong, Hey and van der Molen (1973) as described by Hawkins, Freedman, Marshall and Killen (1975). To each plasma sample (2.5 - 5ml) was added [^{3}H]oestradiol tracer (≃2pg), 50µl 1M sodium hydroxide and the volume made up to 5ml with water. Samples were extracted with 2 x 10ml diethylether and evaporated to dryness. The dried extracts were redissolved in 100µl toluene/methanol (9:1, v/v) and chromatographed on Sephadex LH-20. Quality controls from male rats and man, appropriate blanks and accuracy checks were routinely included. The oestradiol-17β fractions after chromatography were subjected to radioimmunoassay with non-radioactive oestradiol standards. Eluting solvent and appropriate tracer were added to standard curve tubes so as to resemble the sample tubes. After thorough evaporation of solvent the steroid was dissolved in 120µl of ethylene glycol/

0.05M phosphate buffer solution (1:5, v/v), pH 7.0 containing gelatin (2g/l). An aliquot of 20µl was removed for tracer measurement, and 100µl of radioligand solution containing approximately 5,000 c.p.m. (= 13pg) added, followed by 100µl antiserum (raised against oestradiol-17β, 6-carboxymethyloxime conjugated to bovine serum albumin) at a dilution of 1:16,400 with mixing after each addition. Following overnight equilibration at 4°C, free and bound ligand were separated by the addition of dextran-charcoal suspension (0.5g dextran T-70 and 5g Norit A charcoal/litre 0.05M phosphate buffer, pH 7.0). The bound fraction was decanted and its radioactivity measured as in the oestrogen receptor assay. The plasma oestradiol concentration was read from a plot of $\frac{1}{\%\ \text{Bound}}$ vs mass of oestradiol-17β added taking into account assay blanks, recovery losses, and initial plasma volume.

RESULTS

I. Testosterone metabolism by DMBA-induced rat mammary tumours

1. Identification and characterisation of steroids

The metabolism of testosterone by DMBA-induced rat mammary tumours was studied by incubating homogenates of tumour with [7α-^{3}H] testosterone in vitro as described in the METHODS section. After termination of the incubation by the addition of excess methanol, solid material was removed by low-speed centrifugation, and acetone washings of the pellet were bulked with the initial supernatant. The total radioactivity present in the supernatant mixture of incubation medium, methanol and acetone always corresponded closely to the quantity of tritiated precursor originally added to the incubation.

This mixture was evaporated to dryness and the residue redissolved in an ethyl acetate-water partition system. Only the products entering the ethyl acetate phase were routinely investigated, but the quantities of radioactivity present in the aqueous phase of the ethyl acetate-water partition system were also assessed in a limited number of cases.

The evidence for the identification of a radioactive steroid metabolite present in the ethyl acetate fraction was based on the following criteria:-

(1) When subjected to thin layer chromatography the metabolite displayed the same mobility as a sample of the authentic steroid.

(2) The metabolite formed the same derivatives chemically as the authentic steroid under similar reaction conditions. This was checked by observing the change in the mobility of the metabolite and the authentic steroid to that of the expected authentic derivatives in appropriate solvent systems.

(3) When unlabelled authentic steroid (carrier steroid) was added to the products of incubation, the putative metabolite and its different chemical derivatives maintained a constant specific activity on all solvent systems tested.

These criteria were applied in the following manner. The dried residue of the ethyl acetate phase was run in solvent system Ia and the resultant distribution of radioactivity initially examined on the thin layer scanner. Tracings such as those in Fig. 4 revealed that the majority of radioactivity was present in 3 zones, A, B and C. Additional radioactivity was observed sometimes, but not always, in zones D, E, F and G (compare a and b of Fig. 4).

From the examination of the t.l.c. plate under ultra-violet light the positions of the carrier steroids were identified by comparing their mobilities with those of authentic standards run in adjacent channels. The mobilities of the radioactive zones were found to correspond with those of the carrier steroids as follows:- zone A with 5α-androstane-3α,17β-diol and 5α-androstane-3β, 17β-diol which were not separated in this system, R_T (= relative mobility to testosterone) = 0.67; zone B with testosterone, R_T = 1.00 by definition; zone C with 5α-dihydrotestosterone, R_T = 1.29; zone D with 4-androstenedione, R_T = 1.60 and zone E with 5α-androstanedione, R_T = 2.10. Zone F, which was located close to or at the origin, probably contained more polar steroids, but usually represented a very small part of the total radioactivity and was not further investigated. Zone G, R_T = 0.53, was only detected as a separate entity from the adjacent and more radioactive zone A at a late stage in the work. The possible nature of this material is considered at the end of this section.

When zone A was eluted from the initial plate, rerun in solvent system V or VI and scanned, 2 zones of radioactivity were identified (Fig. 5a). The zone with the slightly greater mobility, A_1, ran with authentic 5α-androstane-3α,17β-diol and the other zone, A_2, had the same mobility as 5α-androstane-3β,17β-diol. Both areas were eluted and rerun separately in the same solvent system for further purification. Elution and oxidation of both zones yielded derivatives which ran with the same mobility as 5α-androstanedione in solvent sytem II. (The oxidation of zone A_2 sometimes yielded an extra product which had the same mobility as 4-androstenedione. This area of radioactivity was thought to

Figure 4. Scans of radioactivity in t.l.c. plates - initial plates

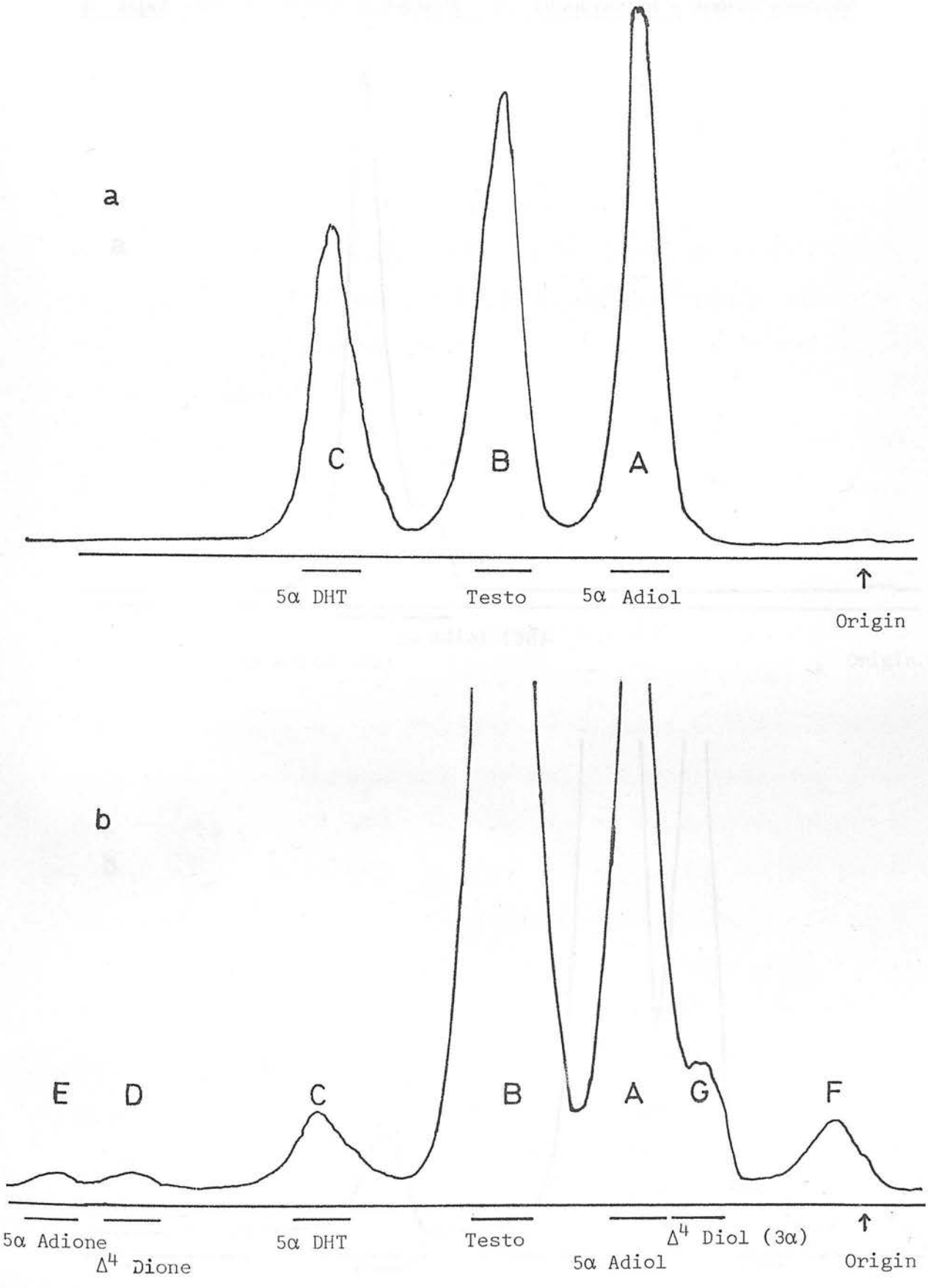
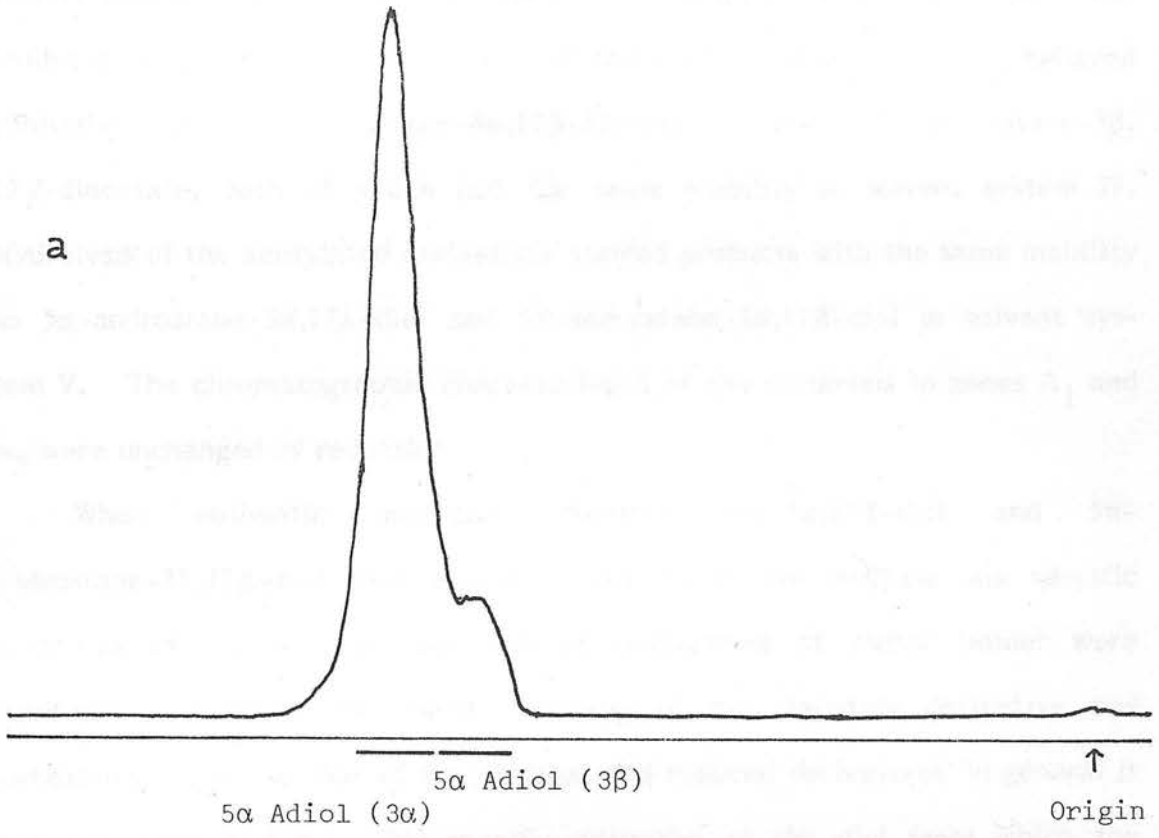
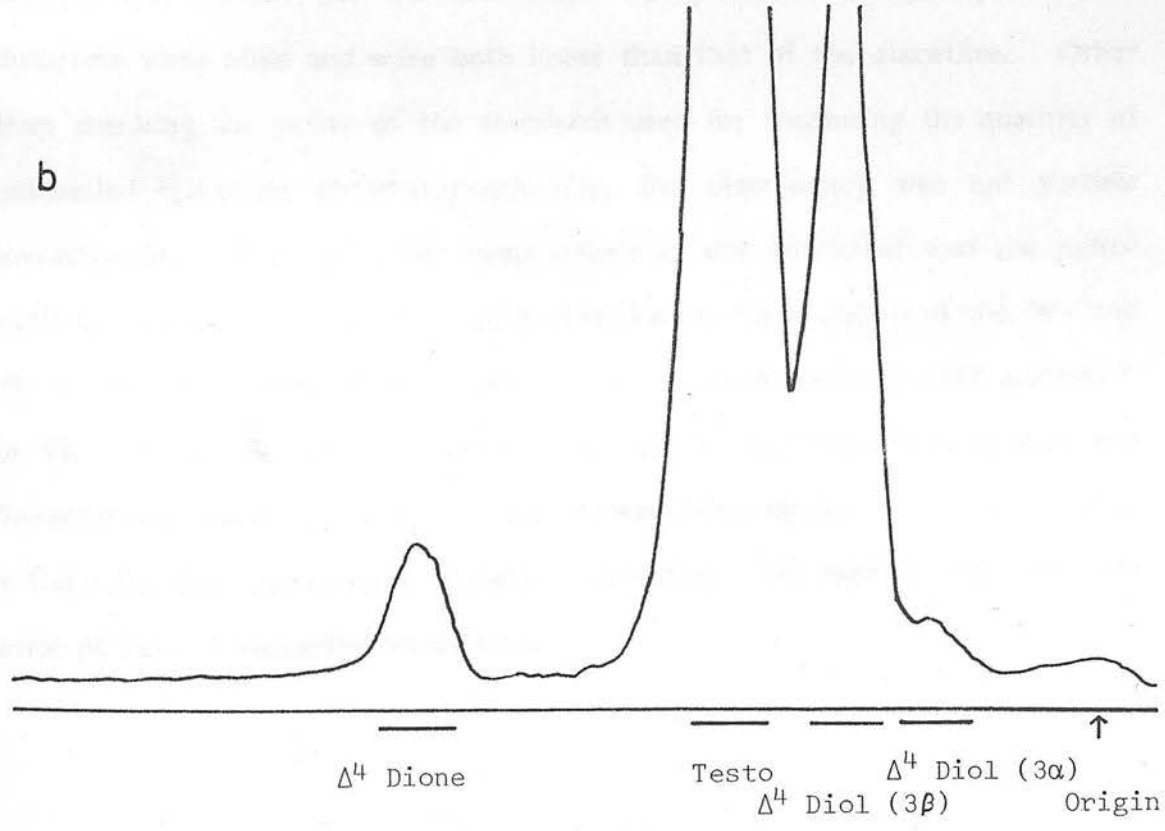


Figure 5. Scans of radioactivity in t.l.c. plates -

a. separation of 5α-androstanediols b. reduction of 4-androstenedione





be due to the oxidation product of contaminant material from zone G of the initial plate.) Subsequent reduction of the oxidised derivatives which had the same mobility as 5α-androstanedione yielded products with the same chromatographic characteristics as 5α-androstane-3β,17β-diol in solvent system III. The products of acetylation of the material from either zones A_1 or A_2 behaved similarly to 5α-androstane-3α,17β-diacetate and 5α-androstane-3β, 17β-diacetate, both of which had the same mobility in solvent system IV. Hydrolysis of the acetylated derivatives yielded products with the same mobility as 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol in solvent system V. The chromatographic characteristics of the materials in zones A_1 and A_2 were unchanged by reduction.

When authentic unlabelled 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were added as carriers to the incubate the specific activities of the oxidised and reduced derivatives of either isomer were identical. Although the specific activity of the diacetate derivative was sometimes similar to that of the oxidised and reduced derivatives, in general it was noticeably higher. The specific activities of the diol from which the acetate was formed and the diol which was produced on hydrolysis of the diacetate were alike and were both lower than that of the diacetate. Other than checking the purity of the standards used for measuring the quantity of unlabelled diacetate chromatographically, the discrepancy was not further investigated. From all other observations it was concluded that the radioactivity in zone A of the initial plate was due to the presence of the 3α- and 3β-isomers of 5α-androstanediol which could be separated in solvent systems V or VI. Since the specific activities of the 5α-androstane-3β,17β-diol and 5α-androstanedione derivatives always showed close agreement, these 2 alone, without the more problematic diacetate derivative, were used for the determination of 5α-androstanediol production.

When the material in zone B of the initial plate was eluted and acetylated the radioactive area had the same mobility as testosterone acetate in solvent system IV. Hydrolysis of the acetylated derivative yielded a product which was chromatographically identical to testosterone in solvent system III. The oxidation of the material in zone B or of the hydrolysate yielded a derivative with the same chromatographic properties as 4-androstenedione in solvent system III. When unlabelled testosterone was added to the incubate as a carrier steroid, constant specific activities were obtained from free testosterone and the oxidised and acetylated derivatives. It was concluded that zone B represented the radioactivity due to unmetabolised testosterone precursor.

Elution and acetylation of the material in zone C of the initial plate yielded a product which was chromatographically identical to 5α-dihydrotestosterone acetate in solvent system VII. Subsequent hydrolysis of this acetate produced a compound with the same mobility as free 5α-dihydrotestosterone in solvent system III. Reduction of the material eluted from zone C or reduction of the hydrolysate yielded products with a chromatographic behaviour identical to that of 5α-androstane- 3β,17β-diol in solvent system III, whereas oxidation of the parent steroid or of the hydrolysate yielded products which had the same mobility as 5α-androstanedione in solvent system II. When unlabelled 5α-dihydrotestosterone was added as a carrier steroid the specific activities of free 5α-dihydrotestosterone and the acetylated, oxidised and reduced derivative remained constant. It was concluded that the steroid in zone C was 5α-dihydrotestosterone.

The mobility of the steroid eluted from zone D did not alter upon acetylation, but remained identical to that of 4-androstenedione in solvent system IV. Likewise the mobility was unaffected by subsequent hydrolysis. Reduction of this steroid under mild conditions (see METHODS) yielded a mixture of 2 main products, one with the mobility of testosterone, the other with

the mobility of 4-androstene-3β,17β-diol in solvent system III (Fig. 5b). Acetylation of the product with the same mobility of testosterone altered its mobility to that of testosterone acetate. Inclusion of unlabelled 4-androstenedione as a carrier steroid resulted in constant specific activities in free steroid, its reduced product testosterone and testosterone acetate. It was concluded that the steroid running in zone D was 4-androstenedione.

Zone E ran close to the solvent front and when reduced ran in solvent system III with the same mobility as 5α-androstane-3β,17β-diol. Oxidation of the reduced product yielded a material with the same mobility as 5α-androstanedione in solvent system II. The chromatographic properties of the original material from the initial plate were unaltered by oxidation or acetylation. When cold 5α-androstanedione was added to the incubate, the specific activities of the free steroid and the reduced derivative remained constant. It was assumed that the radioactivity in zone E was (probably) due to the presence of 5α-androstanedione.

The radioactivity in zone G was first observed as a polar shoulder on the radioactive peak of the 5α-androstanediol band in solvent system Ia. The peak of zone G was more clearly seen when steroids ran further from the origin and it appeared that this peak was greater when those of 5α-dihydrotestosterone were relatively low.

The mobility of zone G (R_T = 0.53) was greater than that of 7α-hydroxytestosterone (R_T = 0.2) and 16α-hydroxytestosterone (R_T = 0.4), and was less than that of 5-androstenediol (R_T = 0.65) and androst-4-ene-3β,17β-diol (R_T = 0.70).

In order to obtain enough material for an analysis of the chemical reactivity of the material in zone G, the areas between the origin and the 5α-androstanediol band from the initial plates of several different incubations were pooled and eluted. When this eluate was rerun in solvent system Ia, 2 peaks were observed, one with the mobility of the 5α-androstanediols and the

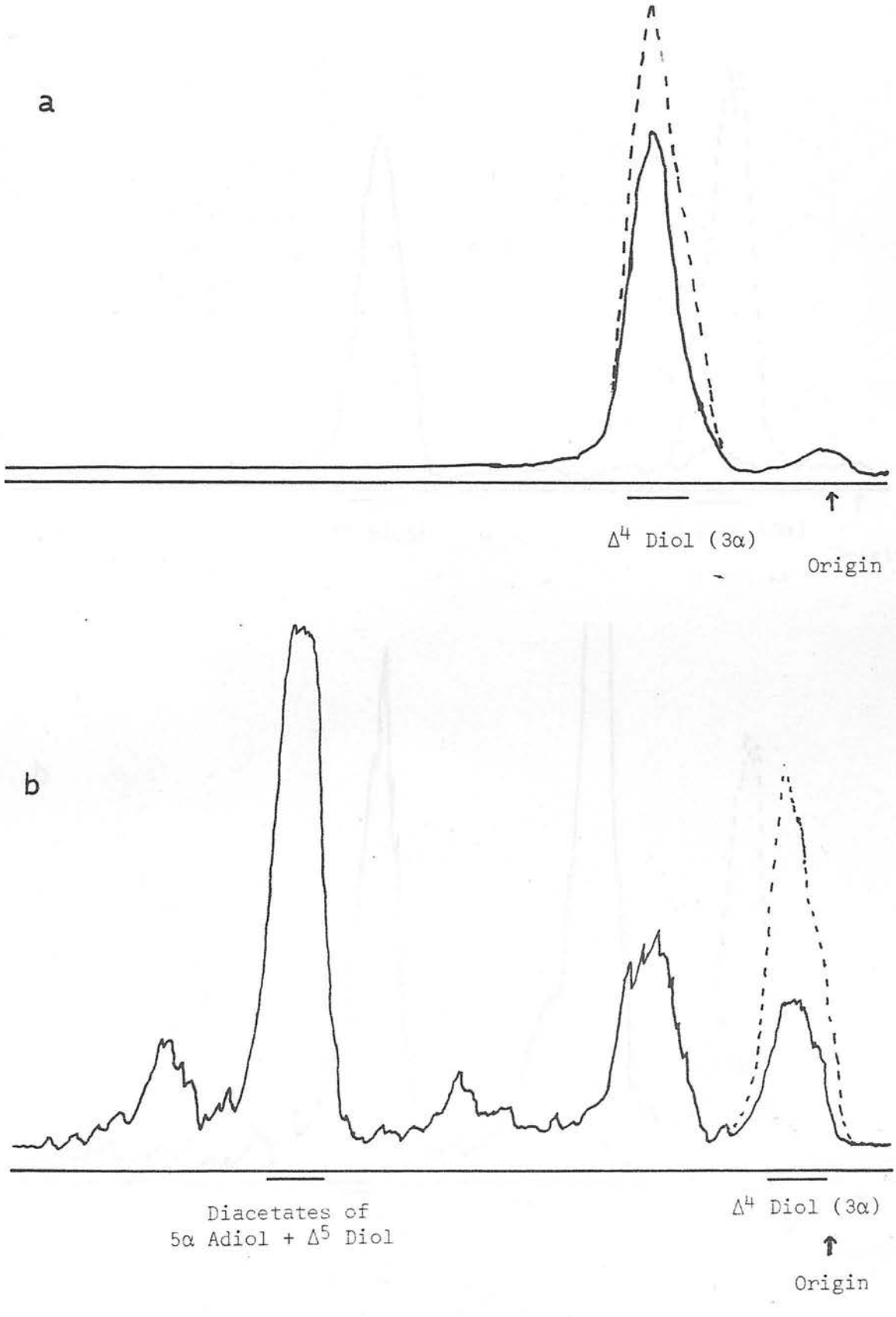
other with the mobility of the original zone G. The material in zone G was recovered and run twice in solvent system Ib to remove contaminant 5α-androstanediol.

When reduction of the material in zone G was attempted there was no alteration in its chromatographic characteristics (Fig. 6a). Acetylation of the material in zone G yielded a major product which had a mobility characteristic of diacetates (e.g. 5α-androstane(3α or 3β)diacetate and 5-androstene-diacetates) in solvent system IV (Fig. 6b). On hydrolysis the mobility of the major acetylated region returned to that of zone G. Oxidation of the material in zone G with chromic acid resulted in a product with the same mobility as 4-androstenedione in solvent system III (Fig. 7a). Reduction of this oxidised product under strong reaction conditions (method a) resulted in a major band of radioactivity, which had the same mobility as androst-4-ene-3β, 17β-diol, and 2 minor bands, one with the same mobility as testosterone and the other with the same mobility as the original zone G in solvent system III. These 3 bands were also seen when weak reduction conditions (method b) were applied to the oxidised product but the radioactivity in the band running with the same mobility as testosterone had increased greatly relative to the other 2. Since authentic 4-androstenedione behaved in the same manner it was concluded that the oxidised product of the material in zone G was probably 4-androstenedione.

The interpretation of these results was that the material in zone G was an unsaturated steroid with 2 hydroxyl groups and no ketone groups. As androst-4-ene-3β, 17β-diol and 5-androstenediol had shown a greater mobility than zone G in the initial solvent system, the possibility of the material in zone G being androst-4-ene-3α, 17β-diol was considered. This steroid was not available commercially, but was later obtained as a gift from Schering. In the meantime the material in zone G was subjected to oxidation by manganese dioxide, a reaction which was reported to show specificity for allylic alcohols (Attenburrow, Cameron, Chapman, Evans, Hems, Jansen and Walker, 1952).

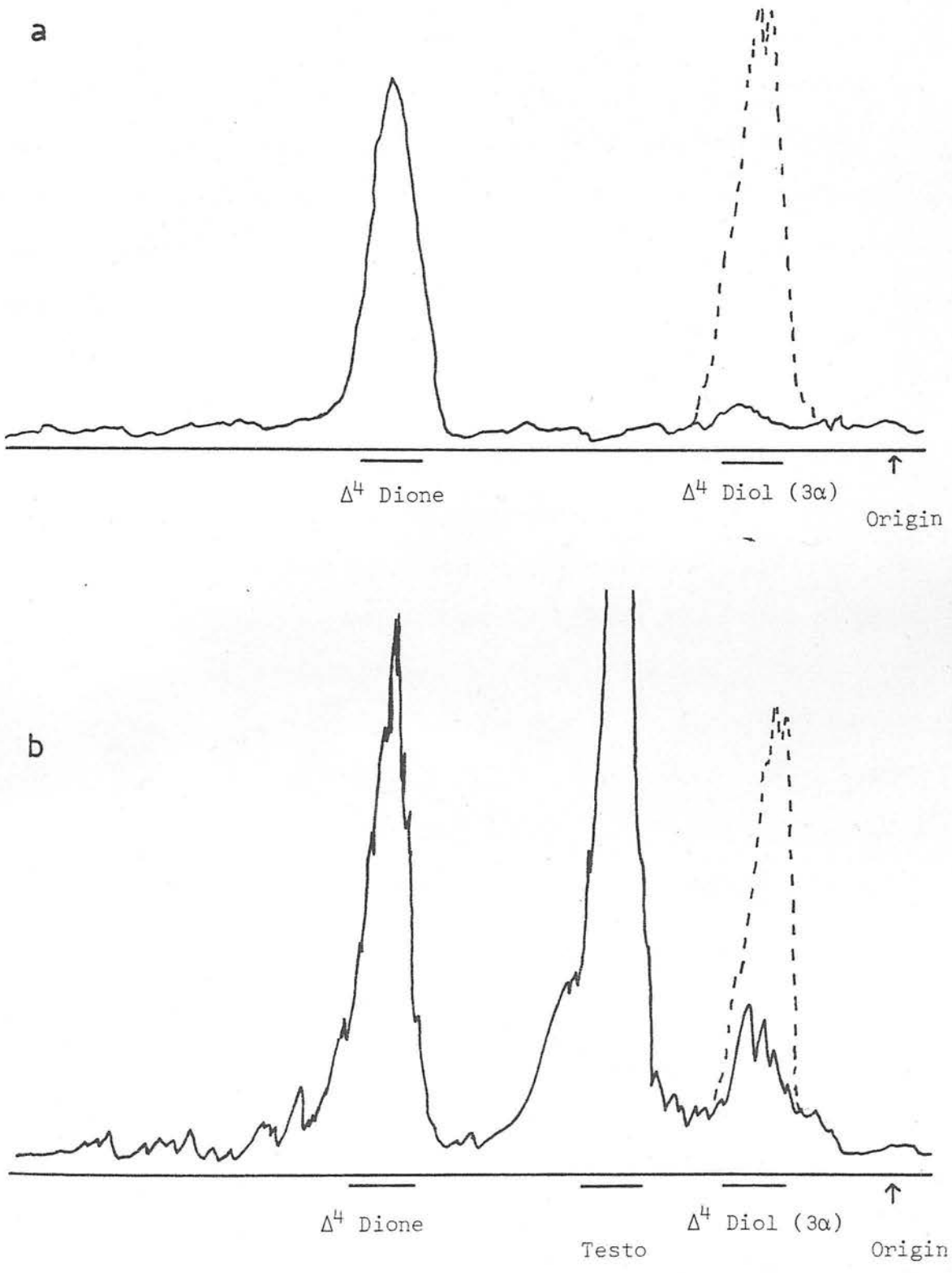
Figure 6. Scans of radioactivity in t.l.c. plates -

a. reduction of zone G b. acetylation of zone G



--- untreated zone G

Figure 7. Scans of radioactivity in t.l.c. plates - oxidation of zone G by a. chromic acid b. manganese dioxide



--- untreated zone G

Three bands of radioactivity were identified when the products of the reaction were run in solvent system III. The major product ran with the same mobility as testosterone (and displayed further properties identical to those of testosterone), and 2 minor bands which ran with the same mobility as 4-androstenedione and the original zone G (Fig. 7b).

When authentic androst-4-ene-3α, 17β-diol was received it was dissolved in ethanol (just soluble at 1mg/ml), and found to be chromatographically pure in all solvent systems tested. Furthermore the chromatographic and chemical behaviour of the authentic steroid was found to be identical to that of the material in zone G. When authentic androst-4-ene-3α, 17β-diol was added as a carrier steroid to a series of incubates (section V), constant specific activities were maintained in 4-androstenedione, testosterone and testosterone acetate derivates. Because of its instability when subjected to g.l.c. the free steroid was not considered as a derivative for the determination of specific activity.

The aqueous phase remaining after the extraction of hydrophobic steroids into the ethyl acetate phase was partitioned twice more with ethyl acetate. The aqueous extract was made up to a volume of 40ml and an aliquot of 0.5ml added to a counting vial containing 10ml of Aquafluor. After an incubation of 2 hours at 37°C, counts were measured until a constant value was obtained. This procedure was performed with the extracts from 12 incubations and, assuming the maximum counting efficiency of 50%, the radioactivity in these aqueous phases accounted for 0.33 - 1.69% of the total radioactivity with a mean of 0.79%. The nature of the radioactivity component(s) of the aqueous phase were not further investigated.

2. Quantification of metabolism

The percentage metabolism of testosterone and its conversion to individual metabolites was determined by measuring the percentage of the total radioactivity which was incorporated into the appropriate steroids. Before extraction, 500µg of non-radioactive carrier steroids were added to monitor recovery

losses and aliquots of the incubates were taken for measurement of the total radioactivity present. Following purification and characterisation of the steroids as described in Table 2 the average specific activity of derivatives was used to estimate the percentage conversion from an equation derived as follows:

$$\% \text{ conversion} = \frac{\text{metabolite formed}}{\text{precursor added}} \times 100$$

$$= \frac{\text{d.p.m. metabolite}}{\text{total d.p.m. incubate}} \times 100$$

$$= \frac{\text{d.p.m. derivative x loss correction}}{\text{total d.p.m. incubate}} \times 100$$

$$= \frac{\text{d.p.m. derivative} \times \frac{\text{nmol carrier added}}{\text{nmol derivative}}}{\text{total d.p.m. incubate}} \times 100$$

$$= \frac{\text{S.A. (ave.) x nmol carrier added}}{\text{total d.p.m. incubate}} \times 100$$

An example illustrating the recoveries of radioactive and non-radioactive steroids and the use of these values to calculate percentage conversion is given in Table 3. Although 5α-androstane-3α,17β-diol was added as a carrier steroid it was the total production of all 4 isomers which was measured since the isomers removed from the initial plate as a 5α-androstanediol band were subsequently oxidised to yield a common product, 5α-androstanedione. This method was routinely employed for determining the steroid conversion described in sections III and IV. When the 3α,17β- and 3β,17β-isomers were added and separated in solvent system V or VI before oxidation, the 3β-isomer was always the minor product, and contributed less than 11% of the total 5α-androstanediols, and less than 3.5% of the total radioactivity, in all incubations of one hour.

Table 3

Details of the measurement of testosterone metabolism

Carrier steroid (nmol)	Derivative	Final solvent system	Part of total	Recovered d.p.m.	Recovered nmol	S.A. (d.p.m./nmol)	Average S.A.	% of total d.p.m.*
Testosterone	testo free	III	1	33,692,992	1,512	22,291		
(1736)	testo acetate	IV	4/5	23,197,432	1,054	22,017	22,137	77.28
	Δ^4dione	III	3/5	16,672,212	754	22,104		
5α-DHT	5α-DHT free	III	$\frac{1}{3}$	357,224	375	952		
(1724)	5α-Adiol(3β)	III	$\frac{1}{3}$	337,693	359	943	949	3.29
	5α-Adione	II	$\frac{1}{3}$	302,006	317	952		
5α-Adiol(3α)	5α-Adiol(3β)	III	$\frac{1}{2}$	2,577,622	681	3,785	3,777.5	13.00
(1712)	5α-Adione	II	$\frac{1}{2}$	2,124,014	563	3,770		

Under each carrier steroid is given the nmol equivalent for the 500μg aliquots added to the incubate.

* total d.p.m. = 49,731,945

The radioactive peaks corresponding to 4-androstenedione and 5α-androstanedione on the initial plate were invariably small or absent. These metabolites were not routinely studied but in the incubations in which their production was measured, that of 4-androstenedione never exceeded 0.68%, n = 13, and that of 5α-androstanedione never exceeded 1.03%, n = 3.

3. Control conditions

In order to determine the non-enzymatic conversion of testosterone to 5α-dihydrotestosterone and 5α-androstanediol a 0.5g portion of tumour was sliced and sonicated, boiled for 15 minutes and then incubated with [^{3}H] testosterone under standard conditions for one hour. The results of this incubation along with those from the incubation of a second portion of the same tumour which was treated normally are shown in Table 4.

The conversion of testosterone to 5α-dihydrotestosterone and 5α-androstanediol was high in the portion of tumour handled normally. In contrast the specific activities associated with 5α-dihydrotestosterone and 5α-androstanediol decreased with sequential derivative formation indicating that the conversion to both steroids under such conditions was less than 0.1%. In accord, unmetabolised testosterone in this incubate accounted for almost all of the radioactivity present.

It can therefore be assumed that the tissue component responsible for the conversion of testosterone to 5α-dihydrotestosterone and 5α-androstanediol is inactivated by heat.

4. Time course of steroid formation

The metabolism of testosterone was studied over a 2 hour period using 3 tumours from different rats, 255L, 269L and 14M. Rat 255L had shown an oestrus-type smear for 3 days and rat 269L had shown a constant oestrus-type vaginal smear for at least 12 days. Rat 14M had been cycling regularly and was in dioestrus. At the time of tumour excision plasma levels of prolactin were 25,

Table 4

Effect of boiling on the ability of a DMBA-induced tumour to metabolise testosterone

Carrier steroid	Derivative	Final solvent system	S.A. (d.p.m./nmol)		% of total d.p.m.
Fresh tissue					
Testosterone	testo free	III	4,547		
	testo acetate	IV	4,297		15.76
	Δ^4-Dione	III	4,302		
5α-DHT	5α-DHT free	III	6,370		
	5α-Adione	II	6,480		22.95
	5α-Adiol(3β)	III	6,425		
5α-Adiol(3α)	5α-Adiol(3β)	III	15,372		53.95
	5α-Adione	II	15,041		
Boiled tissue					
Testosterone	testo free	III	28,481		
	testo acetate	IV	27,405		97.97
	Δ^4-Dione	III	27,793		
5α-DHT	5α-DHT free	III	206		
	5α-Adione	II	139	≤	0.12
	5α-Adiol(3β)	III	35		
5α-Adiol(3α)	5α-Adiol(3β)	III	140	≤	0.12
	5α-Adione	II	36		

45 and 21ng/ml respectively and plasma levels of oestradiol were 0.46, 2.42 and 0.96ng/100ml respectively. All tumours were increasing in size.

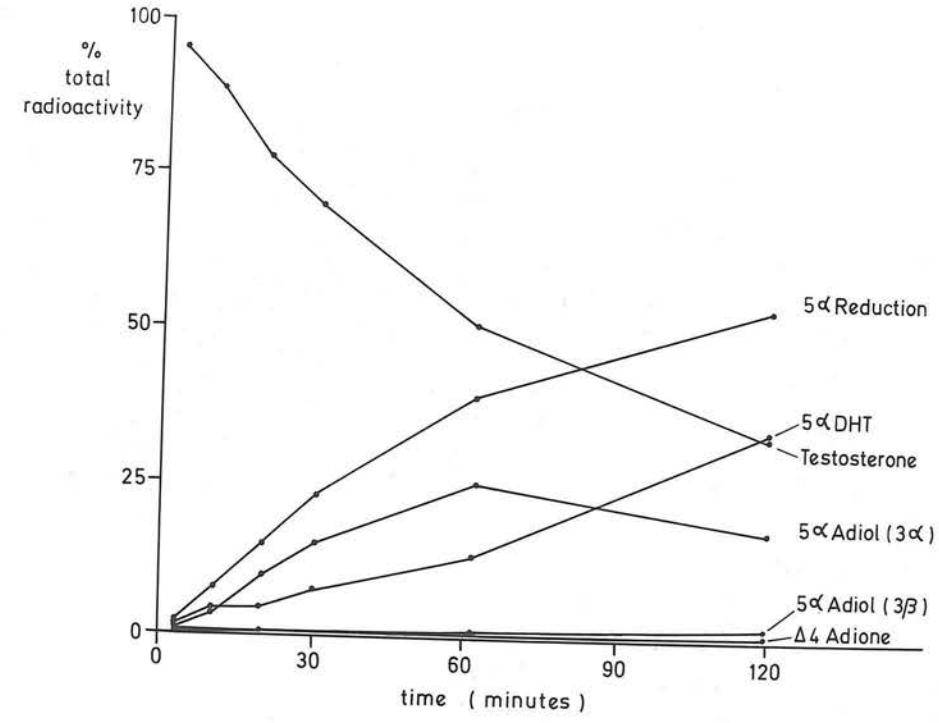
From each tumour a portion of 1.5g was finely sliced and sonicated in 22.5ml Krebs-Ringer phosphate buffer, pH 7.4, to which an NADPH-generating system of 300 μmol glucose-6-phosphate, 45 μmol $NADP^+$ and 75 units glucose-6-phosphate dehydrogenase were added. After a 10 minute preincubation period at 37°C, [^{3}H] testosterone (150 x 10^6 d.p.m.) was added and aliquots of 3.5ml removed into 20ml of ice-cooled methanol at recorded times close to 0, 10, 20, 30, 60 and 120 minutes after testosterone addition. Unlabelled carrier steroids were added to each aliquot and percentage conversions to individual steroids calculated from measurements of the specific activities. In the incubates of 255L RCh and 269L RGr specific activities were determined in only one derivative of each steroid following purification by chemical transformation and t.l.c. The results and details of derivative formation are given with Table 5. The tumour from rat 14M was also used to study the effect of omitting the NADPH-generating system.

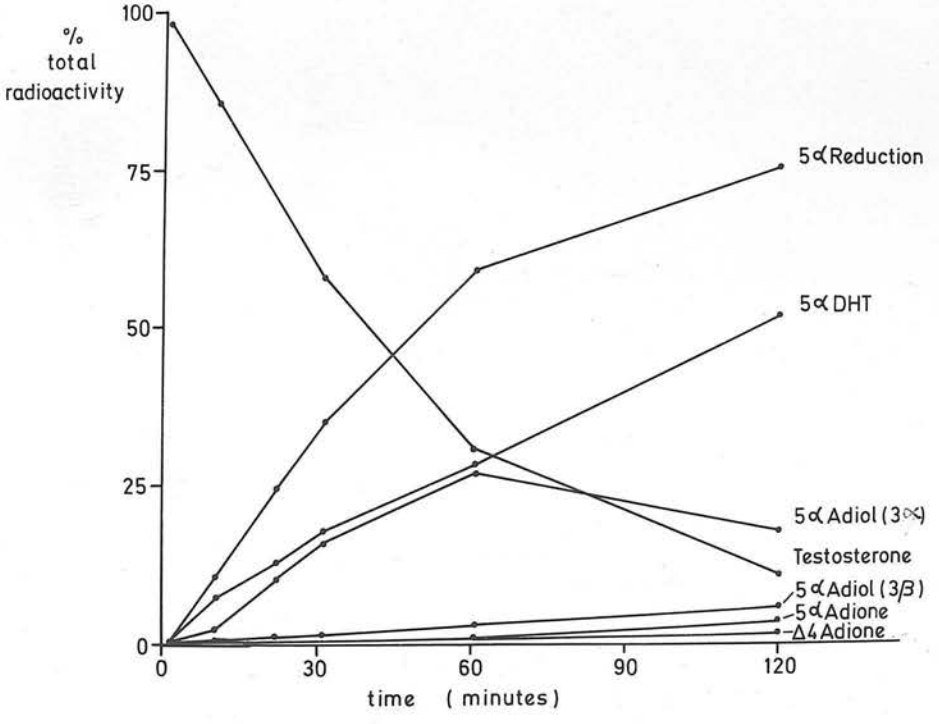
The methods of steroid determination in incubates from 14M LAx, which differed from those mentioned above, are given in the next section along with the results obtained from that tumour (Table 6). The metabolism of testosterone by the 3 tumours is illustrated in Figs. 8 - 10.

It can be seen that in all 3 incubations the metabolism of testosterone was extensive, being accounted for largely by the formation of 5α-reduced products. 5α-Reduction proceeded rapidly for the first hour, but decreased as the level of precursor fell.

Initially the levels of 5α-dihydrotestosterone present in the medium increased more rapidly than those of the 5α-androstanediols, but at 20, 30 and 60 minutes the levels of 5α-androstanediols had exceeded those of 5α-dihydrotestosterone in the incubations of 255L RCh and 14M LAx. Although over the same period the levels of 5α-androstanediols rose more quickly than that of

Figures 8 and 9. Time course of testosterone metabolism by DMBA-induced rat mammary tumours:- 255L RCh, upper; 269L RGr, lower





Legend for Table 5

Preparation of derivatives

Carrier steroid	Reactions for derivative formation	Solvent systems
Testosterone	acetylation	Ia, IV
5α-DHT	acetylation, hydrolysis	Ia, IV, III
5α-Adiol(3α)	oxidation, reduction	Ia, V x 2, II, III
5α-Adiol(3β)	oxidation, reduction	Ia, V x 2, II, III
Δ^4-Dione	"acetylation", "hydrolysis"	Ia, IV, III
5α-Adione	reduction, oxidation	Ia, III, II

Table 5

Time course for the metabolism of testosterone

by DMBA-induced rat mammary tumours

Time in minutes (d.p.m. in aliquot)	Carrier steroid	S.A. (d.p.m/nmol)	% of total d.p.m.
Rat 255L			
3	testosterone	9,356	95.22
(17,057,697)	5α-DHT	135	1.36
	5α-Adiol(3α)	54.7	0.55
	5α-Adiol(3β)	5.63	0.056
	Δ^4-Dione	50.8	0.52
10.5	testosterone	10,316	86.24
(20,764,544)	5α-DHT	492	4.08
	5α-Adiol(3α)	433	3.57
	5α-Adiol(3β)	23.6	0.19
	Δ^4-Dione	57.8	0.48
20	testosterone	7,758	77.31
(17,421,081)	5α-DHT	457	4.52
	5α-Adiol(3α)	982	9.65
	5α-Adiol(3β)	52.3	0.51
	Δ^4-Dione	49.2	0.49
30.5	testosterone	6,730	69.41
(16,831,107)	5α-DHT	721	7.39
	5α-Adiol(3α)	1,453	14.78
	5α-Adiol(3β)	66.3	0.67
	Δ^4-Dione	48.7	0.51

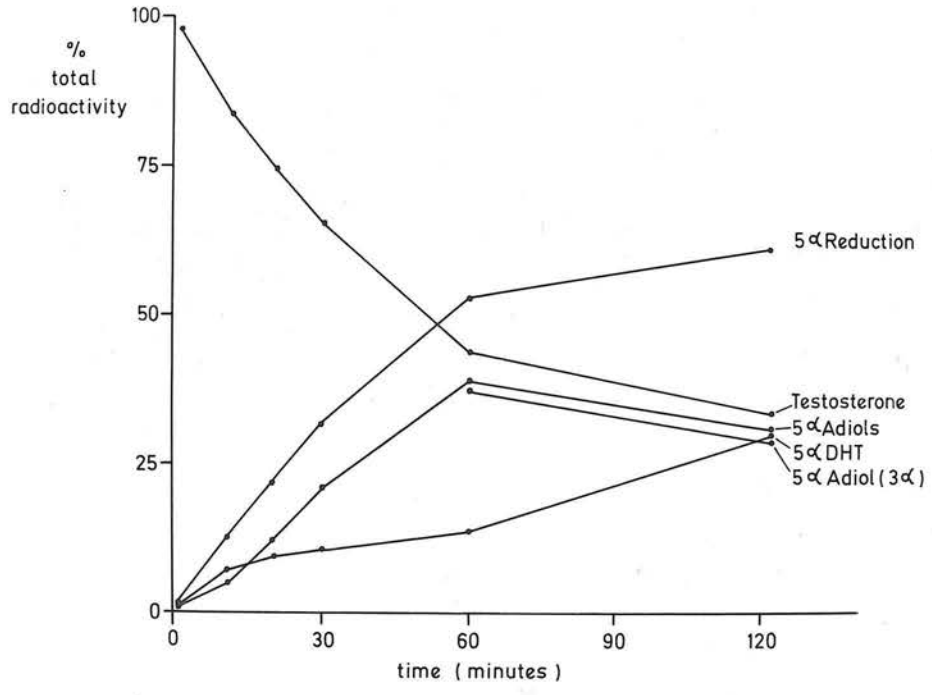
Table 5 (continued)

Time in minutes (d.p.m. in aliquot)	Carrier steroid	S.A. (d.p.m./nmol)	% of total d.p.m.
62	testosterone	4,737	50.36
(16,331,056)	5α-DHT	1,247	13.16
	5α-Adiol(3α)	2,361	24.75
	5α-Adiol(3β)	83.2	0.86
	Δ^4-Dione	52.7	0.56
120	testosterone	3,448	32.42
(18,463,534)	5α-DHT	3,617	33.77
	5α-Adiol(3α)	1,888	17.51
	5α-Adiol(3β)	223.2	2.07
	Δ^4-Dione	77.1	0.73
Rat 269L			
0.5	testosterone	12,764	98.02
(22,468,853)	5α-DHT	77.4	0.59
	5α-Adiol(3α)	5.91	0.045
	5α-Adiol(3β)	1.84	0.014
	Δ^4-Dione	87.0	0.68
	5α-Adione	4.42	0.034
10.5	testosterone	9,638	85.67
(19,530,094)	5α-DHT	879	7.76
	5α-Adiol(3α)	316	2.77
	5α-Adiol(3β)	27.1	0.24

Table 5 (continued)

Time in minutes (d.p.m. in aliquot)	Carrier steroid	S.A. (d.p.m./nmol)	% of total d.p.m.
22	testosterone	7,581	70.71
(18,612,585)	5α-DHT	1,447	13.40
	5α-Adiol(3α)	1,118	10.28
	5α-Adiol(3β)	132	1.21
31	testosterone	6,732	57.59
(20,292,365)	5α-DHT	2,081	17.68
	5α-Adiol(3α)	1,907	16.09
	5α-Adiol(3β)	161	1.35
60.5	testosterone	3,721	30.64
(31,081,479)	5α-DHT	3,505	28.66
	5α-Adiol(3α)	3,355	27.25
	5α-Adiol(3β)	384	3.12
	Δ^4-Dione	126	1.04
	5α-Adione	125	1.03
120	testosterone	1,352	11.10
(21,142,397)	5α-DHT	6,394	52.14
	5α-Adiol(3α)	2,232	18.07
	5α-Adiol(3β)	715	5.79
	Δ^4-Dione	232	1.92
	5α-Adione	419	3.14

Figures 10 and 11. Time course of testosterone metabolism by DMBA-induced rat mammary tumour (14M LAx) in the presence of (upper), and absence of (lower) an NADPH-generating system



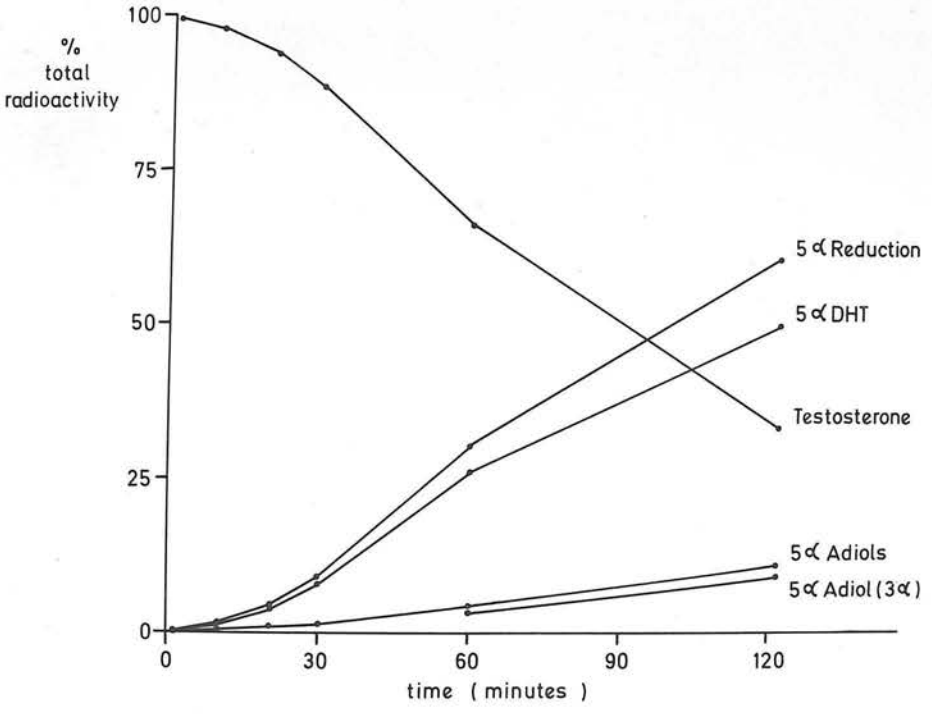


Table 6

Time course of testosterone metabolism by a DMBA-induced mammary tumour (14M LAx), with and without the addition of an NADPH-generating system

Time in minutes (d.p.m. in aliquot)	Steroids	S.A. (d.p.m./nmol)	% of total d.p.m.
With NADPH-generating system			
0.5	testosterone	12,676	97.81
(22,497,408)	5α-DHT	127	0.97
	5α-Adiols	80.4	0.34
10.5	testosterone	9,552	83.44
(19,810,400)	5α-DHT	880	7.66
	5α-Adiols	613	5.30
20.5	testosterone	8,459	74.37
(19,744,766)	5α-DHT	1,114	9.72
	5α-Adiols	1,408	12.21
30	testosterone	8,112	65.29
(21,594,948)	5α-DHT	1,386	11.06
	5α-Adiols	2,622	20.78
60	testosterone	4,837	43.81
(19,166,122)	5α-DHT	1,552	13.96
	5α-Adiols	4,348	38.84
	as 5α-Adiol(3α)	4,157	37.14
122	testosterone	4,561	33.54
(23,604,646)	5α-DHT	4,102	29.96
	5α-Adiols	4,291	31.12
	as 5α-Adiol(3α)	4,022	29.17

Table 6 (continued)

Time in minutes (d.p.m. in aliquot)	Steroids	S.A. (d.p.m./nmol)	% of total d.p.m.
Without NADPH-generating system			
0.5	testosterone	9,788	99.33
(17,106,056)	5α-DHT	15.5	0.16
	5α-Adiols	28.9	0.29
10	testosterone	8,006	97.91
(14,195,325)	5α-DHT	94.7	1.15
	5α-Adiols	28.2	0.34
20.5	testosterone	7,735	93.97
(14,289,491)	5α-DHT	302	3.64
	5α-Adiols	39.3	0.47
30	testosterone	8,311	88.42
(16,022,250)	5α-DHT	725	7.66
	5α-Adiols	110	1.15
60	testosterone	7,345	66.06
(19,300,708)	5α-DHT	2,908	25.98
	5α-Adiols	473	4.20
	as 5α-Adiol(3α)	378	3.26
122	testosterone	3,349	33.28
(17,469,234)	5α-DHT	5,071	50.04
	5α-Adiols	1,119	10.97
	as 5α-Adiol(3α)	982	9.62

5α-dihydrotestosterone in the incubation of 269L RGr, only at 60 minutes did the combined level of the 3α- and 3β-isomers of 5α-androstanediol exceed that of 5α-dihydrotestosterone.

Between 60 and 120 minutes the levels of 5α-androstane-3α,17β-diol, but not those of the 3β-isomer, fell, and the levels of 5α-dihydrotestosterone increased in all 3 incubations. Since the increases in 5α-dihydrotestosterone levels between 60 and 120 minutes were greater than the corresponding decreases in the levels of testosterone, one must conclude that during this period, conversion of 5α-androstane-3α,17β-diol back to 5α-dihydrotestosterone had occurred. There was no evidence for the conversion of 5α-androstane-3β,17β-diol to 5α-dihydrotestosterone.

It was noticed that the levels of 4-androstenedione recovered initially were relatively high in the incubations of 255L RCh (0.55%) and 269L RGr (0.68%), but increased very slowly. The batch of precursor which was used for both these incubations had been purified 2 weeks prior to use and was stored at 4^{o}C. When these results were finally calculated, about 8 weeks after incubation, the original batch of precursor was still available since it had not been required for further incubations and therefore not repurified. An aliquot of the precursor solution, which had now been purified 10 weeks previously, was mixed with 100μg of unlabelled testosterone and 100μg unlabelled 4-androstenedione. These steroids were separated in solvent system III. After correcting for losses from the amount of unlabelled steroid recovered the distribution of radioactivity was as follows:- testosterone - 98.72%, 4-androstenedione - 0.82% and the remainder of the plate 0.46%. It therefore seems likely the[^{3}H]4-androstenedione found in the early stages of incubation did not arise by enzymatic conversion of testosterone but was probably present in the aliquot of precursor added.

5. Effect of an NADPH-generating system on testosterone metabolism

The 5α-reduction of testosterone to 5α-dihydrotestosterone and 5α-androstanediol required a supply of hydrogen ions. NADPH provides a suitable source

of hydrogen ions (Moore & Wilson, 1975), and therefore a system which generates NADPH was routinely included in the incubations performed for this thesis. The results in this section show the effect of omitting the NADPH-generating sytem on the metabolism of testosterone over a 2 hour incubation period.

Tumour 14M LAx was divided into 2 portions of 1.5g. The first portion was incubated with an NADPH-generating system containing $NADP^+$, glucose-6-phosphate and glucose- 6-phosphate dehydrogenase as described in the previous section. All 3 components were omitted from a parallel incubation of the second portion of tumour. The standard methods of steroid determination were modified to the following procedure. Unlabelled testosterone, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol (500μg of each) were added to all aliquots removed from the 2 incubations. The steroids extracted in the organic phase were run in solvent system Ib, scraped off, eluted and rerun separately in solvent system III. The specific activities were determined in the bands of steroid removed from this plate. Since the 3α- and 3β- isomers of 5α-androstanediol run identically in these systems the specific activity of the band with the 5α-androstane-3α,17β-diol carrier steroid reflected the radioactivity associated with total 5α-androstanediol levels. The isomers of 5α-androstanediol were then separated in solvent sytem V and the specific activity of the 3α-isomer determined. The specific activities and corresponding percentage conversions are given in Table 6 and the latter shown diagrammatically in Figs. 10 and 11.

The pattern of steroid metabolism by the portion incubated with the NADPH-generating system resembled those of the 2 tumours described in the previous section and is discussed along with them.

When the NADPH-generating system was omitted the pattern of testosterone metabolism was considerably altered, showing a slow initial rate of metabolism which increased up to 60 minutes and stayed high for the remainder of the incubation period. This meant that at 60 minutes almost twice as much testosterone had been metabolised in the incubation to which an NADPH-

generating system had been added, but after 120 minutes similar amounts of testosterone had been metabolised in both incubations.

Although in the absence of an NADPH-generating system testosterone metabolism was still largely accounted for by 5α-reduction, the composition of the 5α-reduced metabolites was the reverse of that normally found. At all times the levels of 5α-dihydrotestosterone greatly exceeded those of the 5α-androstanediols. Moreover, there was no decrease in the levels of 5α-androstane-3α, 17β-diol between 60 and 120 minutes.

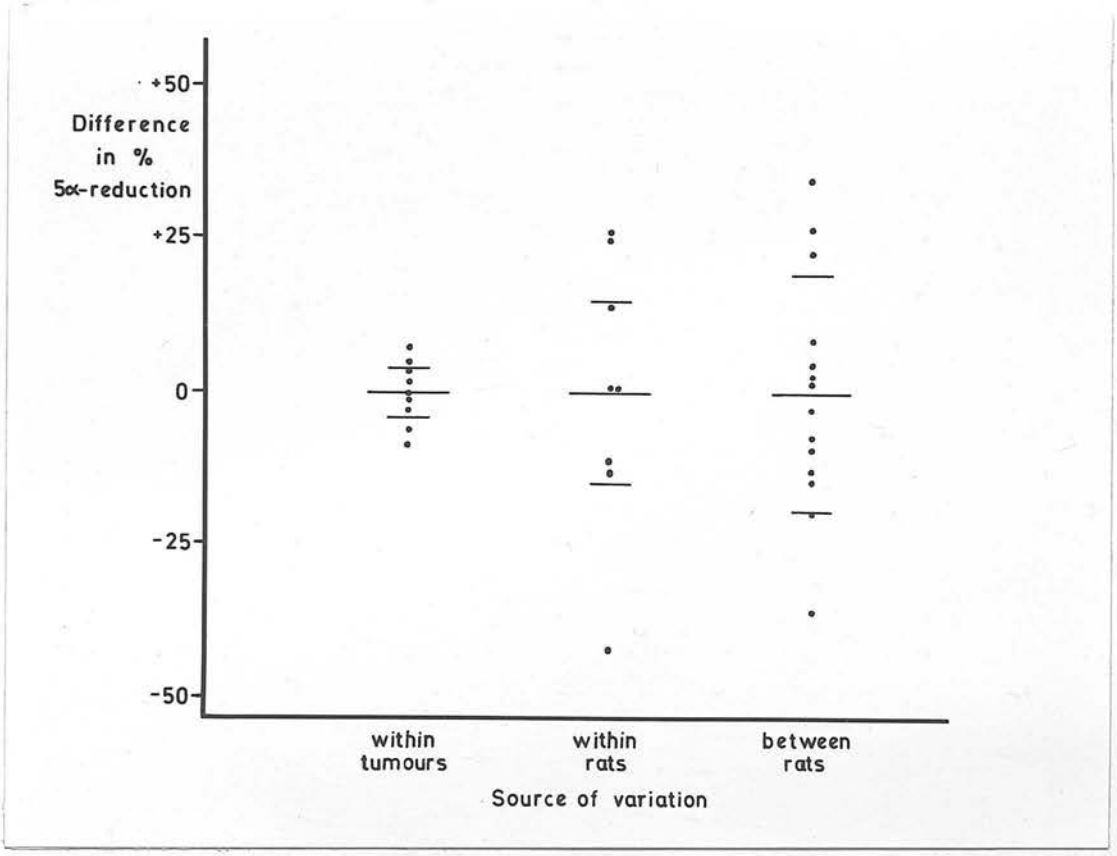
The different method used to measure 5α-androstane diol production in these incubates gave results in close agreement with the conventional methods used in the previous section, namely, that in both series of incubates, the contribution of the 3β-isomer to the total 5α-androstanediol was small and that in the incubation containing the NADPH-generating system the level of 3α-isomer, but not that attributed to the 3β-isomer (total 5α-androstanediol minus the 3α-isomer), declined between 60 and 120 minutes.

6. Source of variation in the measurement of testosterone metabolism

To ascertain the precision of method used to measure testosterone metabolism duplicate portions of certain larger tumours were separately incubated under the same standard conditions. In a few cases incubations were performed on 2 tumours taken from one rat. The data from these incubations have been used to compare variations within tumours, between tumours within rats and between rats. The parameter of 5α-reduction which accounts for the majority of testosterone metabolised has been considered.

The tumours used for duplicate incubations came from 6 untreated and 3 perphenazine-treated rats. For the assessment of variation between 2 tumours from the same rat, results from untreated rats only have been used. The averages of the duplicate portions from the 6 untreated rats and the values of the "a" tumours from rats with pairs of tumours were used to assess the variation between rats (Table 7).

Figure 12. Variation in the 5α-reduction of testosterone within and between DMBA-induced mammary tumours



The points in the first two columns represent the difference between pairs of values minus the mean of the differences. The points in the third column represent individual values minus the mean. Horizontal bars indicate means (all zero after the above transformation) and s.d.'s (see text).

Table 7

Variations in the 5α-reduction of testosterone

by DMBA-induced mammary tumours

Source of variation	5α-reduction % a	b	a - b	$\frac{a+b}{2}$		
	32.17	30.59	+ 1.58	31.38*		
	49.58	49.10	+ 0.48	49.34*		
	36.99	30.43	+ 6.56	33.71*		
portions	47.42	39.51	+ 7.91	43.46*	$\overline{(a-b)}$	= +2.94
of same	5.87	3.11	+ 2.76	4.49*	$\bar{R}$	= 4.74
tumour	64.77	70.06	- 5.29	67.42*	s.d. (est)	= 4.1
	81.93	71.46	+10.47	from		
	41.66	36.84	+ 4.82	perphenazine-		
	30.56	33.40	- 2.84	treated rats		
	37.80*	41.72	- 3.92			
	45.48*	35.84	+ 9.04			
tumours	43.00*	22.17	+20.83		$\overline{(a-b)}$	= -4.31
of same	27.62*	44.92	-17.30		$\bar{R}$	= 17.42
rat	21.11*	67.42	-46.31		s.d. (est)	= 14.9
	26.16*	30.13	- 3.97			
	75.57*	53.62	+21.95			
	63.83*	79.24	-15.41			
tumours of different rats	values marked * above used				mean	= 40.74
					s.d.	= 19.21

mean of range, $\bar{R} = \overline{|a-b|}$, s.d. est $= \frac{\bar{R}}{d_2}$ (see text)

The 5∝-reduction from the arbitrarily designated "b" incubates were subtracted from those of the corresponding "a" incubates. For diagrammatic comparison of the variation, these values were transformed by subtracting the mean of each set from each individual value within the set. By this transformation the absolute spread of the values remained the same, but the new means were both equal to zero (Fig. 12). The variation between rats was illustrated subtracting the mean 5∝-reduction from individual values. The variation which occurred between pairs of values is expressed in Table 7 by $\bar{R}$, the mean of the range of differences, irrespective of sign. The standard deviation was estimated from the expression $\frac{\bar{R}}{d_2}$ where d is a factor dependent on the degrees of freedom.

The mean of the range of differences between portions of the same tumour was 4.74% with an estimated standard deviation of 4.1 whereas the mean of the range of variation between tumours of the same rat was 17.42 with an estimated standard deviation of 14.9. The standard deviation of 5∝-reduction values obtained from tumours from different rats was 19.2.

This analysis indicates that although certain variations occur between 2 portions of the same tumour, whether due to tumour heterogeneity or limits in methodological precision, the results obtained are largely reproducible. In contrast, the variation between tumours, either within the same rat or in different rats, is far greater. The variation in 5∝-reduction between tumours from the same rats, although a little less than that of tumours from different rats, was considered great enough to include 2 tumours from the same rats as separate entities for intergroup comparison in sections III and IV.

II. Effects of treatment regimes on plasma hormone levels

The main part of this work involved the alteration of the circulating levels of hormones known to be involved in the growth of DMBA-induced mammary tumours so that relationships between tumour steroid metabolism, tumour growth and hormone levels could be studied. The effects of altering plasma prolactin levels in the presence and absence of ovarian influences were particularly investigated. However, since repeated induction of anaesthesia for removal of blood for plasma prolactin assay presented a risk of untimely death and might have altered the levels of hormones which respond to stress, it was proposed to measure plasma prolactin only before and at the end of treatment in rats bearing tumours under study. In addition, since a minimum of 2.5ml of plasma was required for reliable estimation of oestradiol levels in the rat by radioimmunoassay, a single sample of blood for this purpose could only be routinely obtained at sacrifice. Because of these restrictions it was necessary to establish that the chosen treatment regimes were having the desired effect and moreover that hormone levels at sacrifice might appropriately reflect changes due to treatment. Animals which had received DMBA but which had not developed continuously growing tumours were kept under identical conditions to those used in tumour studies. Thus at the same age as their tumour-bearing counterparts these animals provided a suitable stock in which the effect of the various treatment regimes on plasma prolactin and oestradiol could be more elaborately studied. The animals in this category were required to have regular oestrous cycles (4 days), as assessed by vaginal smears, prior to allocation to starting treatment on the day of dioestrus.

1. Perphenazine and plasma prolactin

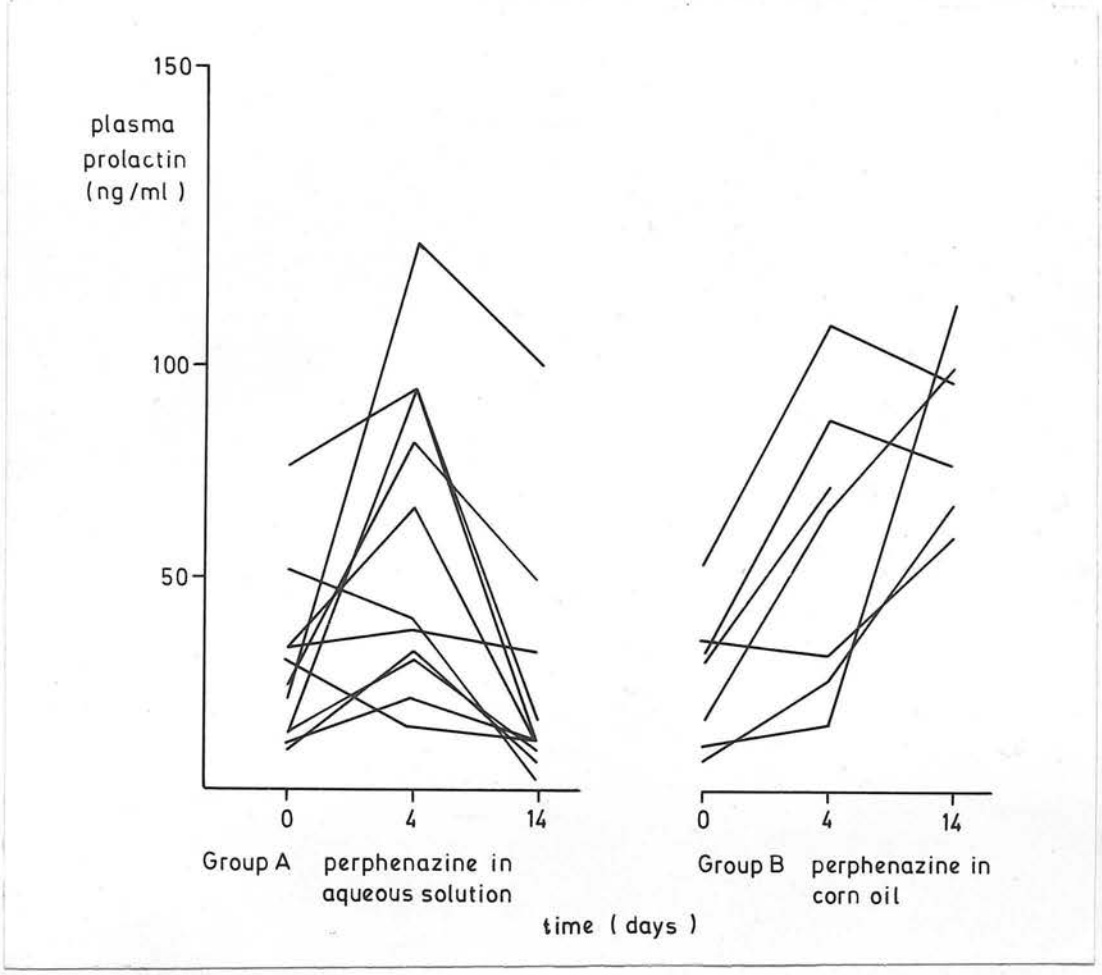
Initially attempts were made to elevate plasma prolactin by daily s.c. administration of an aqueous preparation of perphenazine supplied in ampoules

(5mg/ml) by Allen & Hanburys Ltd. Rats were given 0.2ml daily for 14 days. Plasma prolactin values measured 24 hours after the 4th and 14th injection are shown in Fig. 13. Most, but not all, animals showed an increase in plasma prolactin at 4 days but by 14 days plasma prolactin had fallen in all animals, in some cases to levels below pre-treatment values. A comparison of the prolactin values in this group by the paired t-test (Table 8) revealed that while levels were significantly elevated on day 4 of treatment there was no significant difference between pretreatment levels and those on day 14.

In view of the inability of the commercial preparation to sustain elevated plasma prolactin levels it was decided to change the vehicle. Perphenazine powder (Allen & Hanburys Ltd) was dissolved in corn oil (5mg/ml) and daily s.c. injections (1ml/kg body weight) given to 7 rats. Blood was again taken before and 4 and 14 days after treatment. Plasma prolactin levels, also shown in Fig. 13, were elevated in 6 out of 7 animals at 4 days and in all 6 surviving animals at 14 days. Group analysis shows that prolactin levels were significantly higher at 4 and 14 days compared to pretreatment levels (Table 8).

This regime was therefore studied in more detail and Fig. 14 shows mean prolactin values for the intermediate days in 6 rats so treated. An increase in plasma prolactin was evident 24 hours after the first injection and had reached maximal levels by day 2. Although the mean value from day 2 onwards remained relatively constant there were individual variations in the plasma levels. Prolactin levels in a group of animals given only corn oil vehicle are shown in Fig. 18. The mean prolactin values in this group are approximately half those of the perphenazine treated group and show fluctuation which probably represents variation due to the oestrous cycle. From the vaginal smear pattern it appeared that perphenazine treatment arrested the normal oestrous cycle presenting most commonly with many leukocytes as in a dioestrus-type smear after 1 - 4 days of perphenazine treatment.

Figure 13. Effect of two perphenazine preparations on plasma prolactin levels



Rats in group A received a daily s.c. injection of 0.2ml Allen & Hanburys' aqueous fentazin preparation (5mg/ml). Rats in group B received a daily s.c. injection of 1ml/kg body weight of perphenazine B.P. dissolved in corn oil. Plasma samples were obtained 24 hours after perphenazine administration.

Table 8

Effects of two perphenazine preparations on plasma prolactin levels

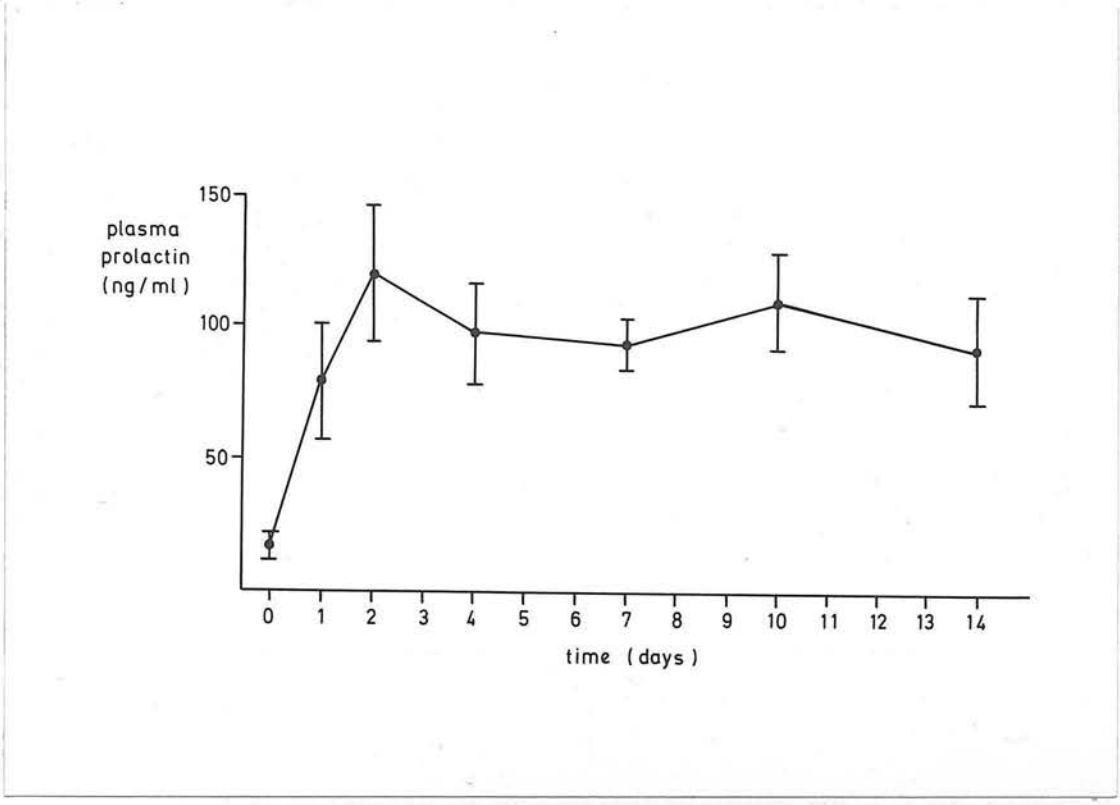
Perphenazine preparation (daily dose)	Number of rats	Plasma prolactin (ng/ml) Pretreatment 0	Days of treatment 4	14
Fentazin in aqueous solution (1mg/rat)	11	28.4 ± 6.2[a]	57.9 ± 11.1 ($p < 0.025$)[b]	21.6 ± 8.3 N.S.
Perphenazine in corn oil (5 mg/kg body weight)	7	26.1 ± 6.1	58.6 ± 13.3 ($p < 0.01$)	84.5 ± 7.63 ($p < 0.0025$)

The aqueous solution of Fentazin (perphenazine) was obtained from ampoules for injection prepared commercially by Allen & Hanburys Ltd.

a = mean ± s.e. mean

b = obtained by comparison with pretreatment values by t-test for paired samples.

Figure 14. Effect of daily administration of perphenazine on plasma prolactin levels



Each point represents the mean of the plasma prolactin levels in 6 rats which received a daily s.c. injection of perphenazine (5mg/kg body weight) in corn oil solution. Plasma samples were obtained 24 hours after injection. Vertical lines indicate s.e. means.

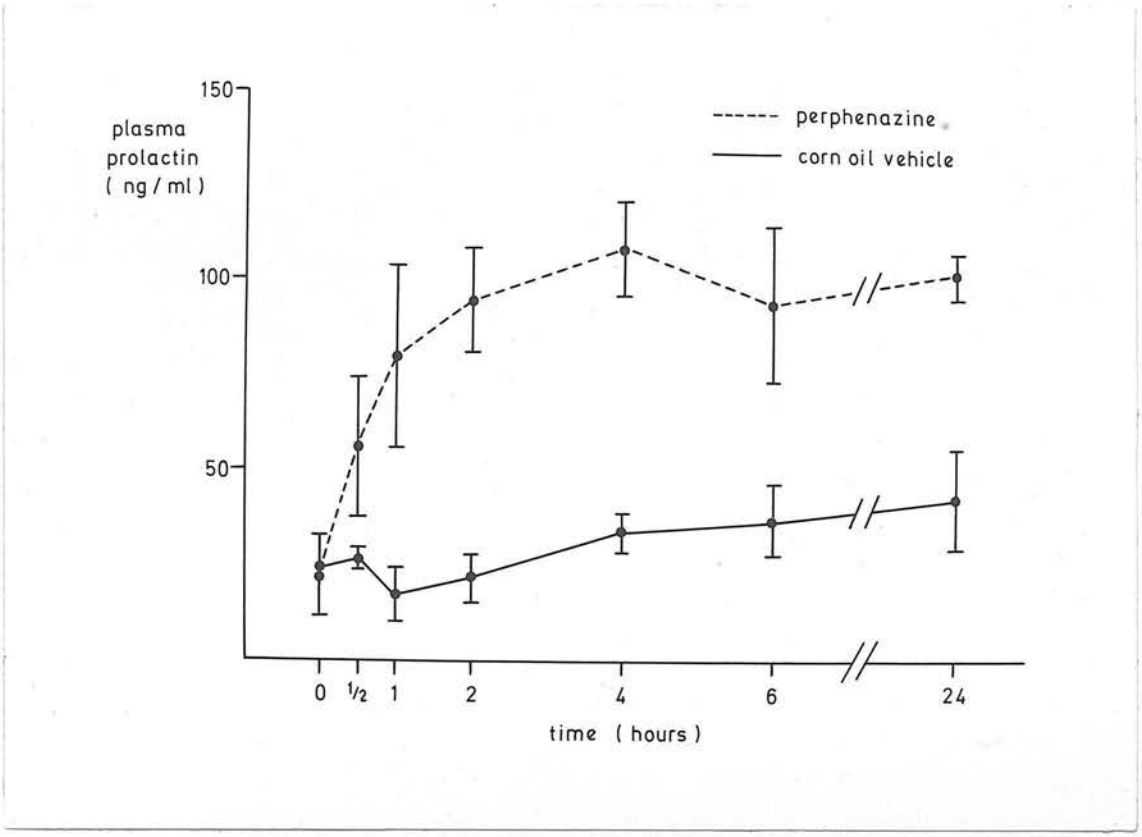
The effect of a single injection of perphenazine in corn oil at 1200 hours on the day of dioestrus in 8 rats is shown in Fig. 15. Administration of the drug produced a rapid rise in plasma prolactin, elevated levels being detected within 30 minutes in certain individuals, and all animals showed maximally raised levels by 4 hours. Plasma prolactin levels remained significantly elevated even 24 hours after the administration of perphenazine. Control animals given corn oil vehicle failed to show such a marked rise in plasma prolactin although there was a slight trend of increased prolactin levels up to 24 hours, possibly due to rats moving into the proestrus stage of the cycle or due to stress.

It was also of interest to know the effect of a single injection of perphenazine when prolactin levels were already elevated by previous injections of perphenazine. Blood samples were therefore collected from 6 rats during the 24 hour period after the 12th and final daily injection of perphenazine. The plasma prolactin levels in these samples are shown in Fig. 16. The mean plasma prolactin level just prior to the final injection (i.e. 24 hours after the 11th injection) was significantly higher than the pretreatment value. Although there was a trend towards increased prolactin levels 2 hours after the final injection the large scatter in values renders the difference insignificant. It is apparent from these results that the measurement of plasma prolactin 24 hours after the final injection of perphenazine should provide a reasonable indication of the mean prolactin levels over the period prior to sacrifice. The measurement of plasma prolactin in single sacrifice blood samples was used to assess the efficacy of perphenazine in the experimental protocols for tumour-bearing rats.

2. Ovariectomy and plasma prolactin

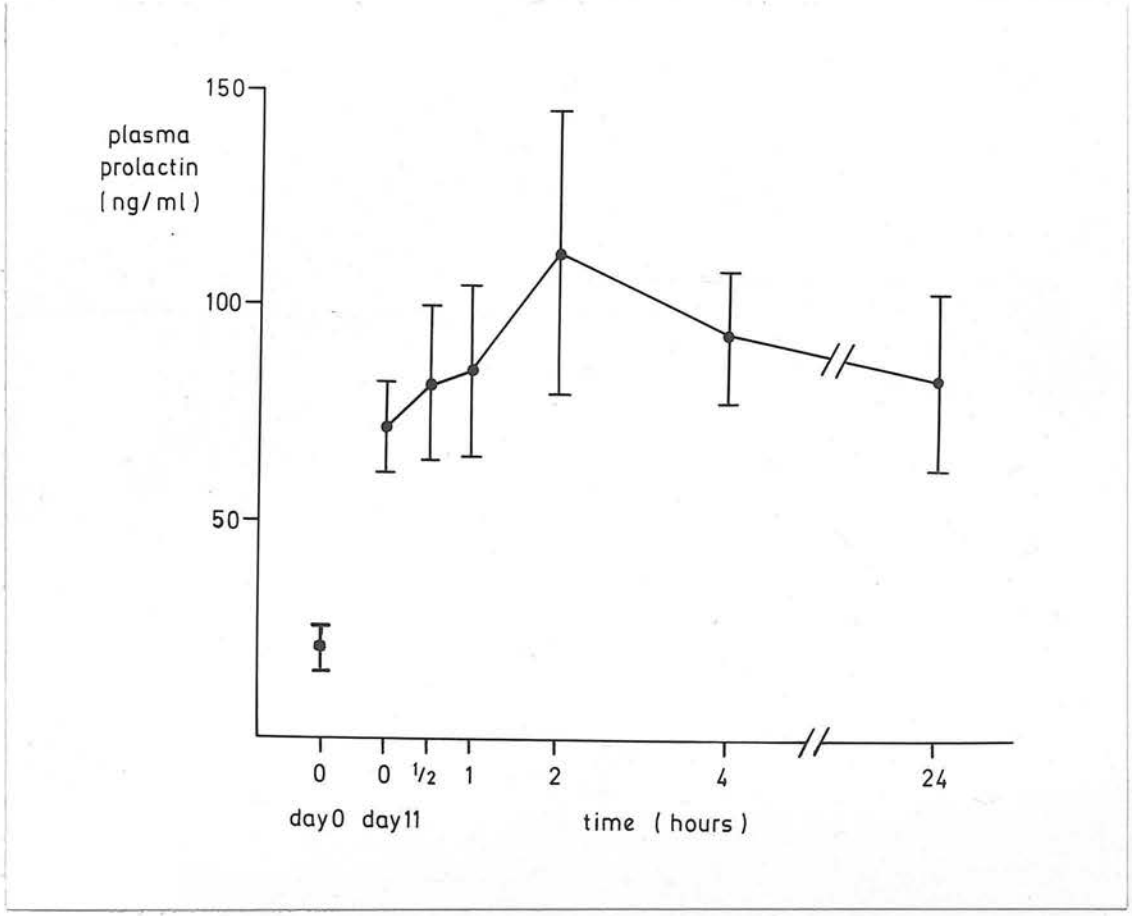
In order to determine the effects of ovariectomy on plasma prolactin levels, blood was taken from 4 animals immediately before and at 2 and 4 hours and 1, 2, 4, 8 and 16 days after ovariectomy. As controls 4 animals were sham-operated and blood taken at the same time. The results are shown in Fig. 17. Since the operations were performed on the day of dioestrus in animals with

Figure 15. Plasma prolactin levels after a single dose of perphenazine



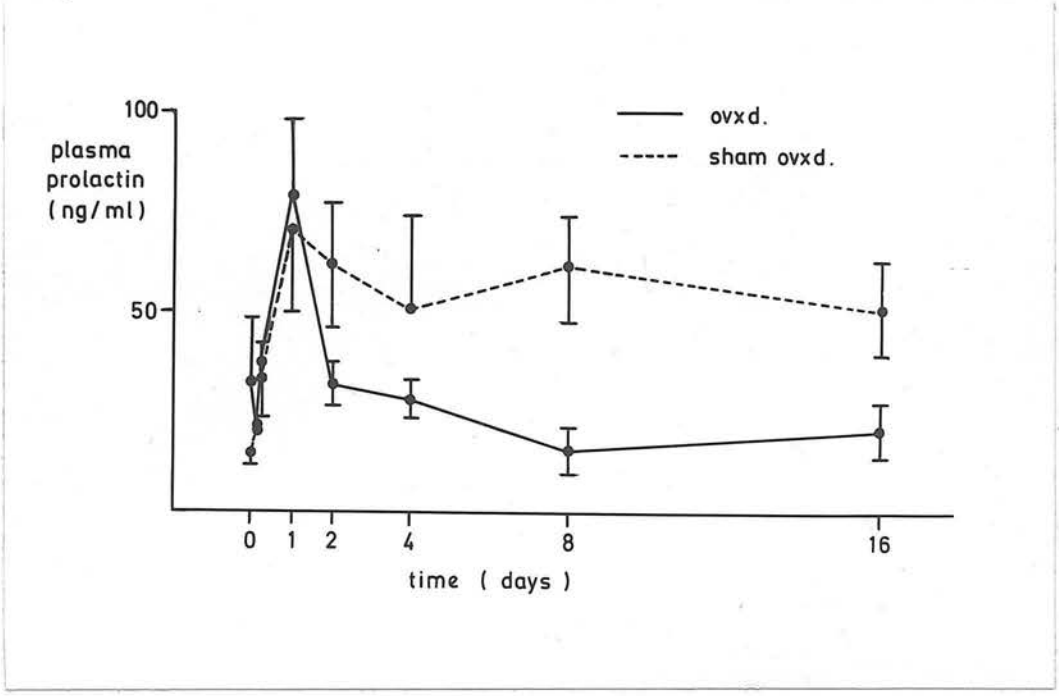
At noon of dioestrus (time = 0) rats received a single s.c. injection of (a) perphenazine, 5mg/kg body weight, n = 8; or (b) corn oil vehicle, 1ml/kg body weight, n = 6. Vertical lines indicate s.e. means.

Figure 16. Plasma prolactin levels after the 12th dose of perphenazine



The 12th daily s.c. injection of perphenazine (5mg/kg body weight) was given to a group of rats (n = 6) at noon on day 11 of treatment. Vertical lines indicate s.e. means.

Figure 17. Effect of ovariectomy on plasma prolactin levels



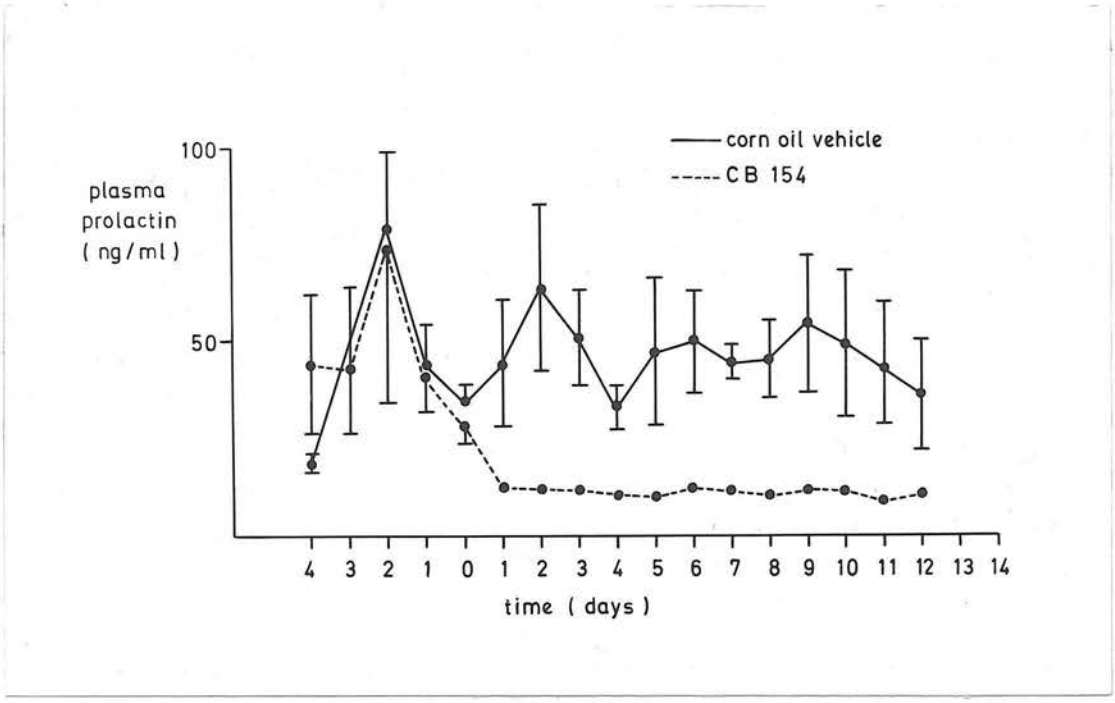
Rats were ovariectomised (n = 4) or sham-operated (n = 4) between 1100 and 1200 hours on the day of dioestrus (day 0). Vertical lines indicate s.e. means.

a 4-day oestrous cycle, operated rats were usually in dioestrus on days 4, 8 and 16. Ovariectomised rats displayed constant dioestrus-type vaginal smears. 24 hours after the operation the mean plasma prolactin levels in both groups were significantly higher than the preoperative values. In the ovariectomised group prolactin levels fell in comparison to those of the sham-operated group and on days 8 and 16 the mean plasma prolactin levels in the ovariectomised group were significantly lower than those in the sham-operated group ($p < 0.01$ and $p < 0.05$ by the Student t-test).

3. CB 154 and plasma prolactin

The ergot alkaloid CB 154 was chosen to suppress the release of pituitary prolactin. The effect of daily administration of this drug is compared with that of the corn oil vehicle in Fig. 18. Daily tail vein blood samples were taken between 1400 and 1500 hours immediately prior to the next injection of drug or vehicle. Plasma prolactin levels were similar in samples taken from both groups during the oestrous cycle immediately prior to starting treatment. In the control animals plasma prolactin levels remained within the same range as pretreatment values and although there was considerable variation between individual levels there appears to be a cyclic variation in plasma prolactin. In contrast plasma prolactin levels were greatly suppressed by CB 154 treatment with no indication of cyclic variation. From day 1 of treatment onwards prolactin levels were always significantly lower in the CB 154 treated animals ($p < 0.05$ by the Student t-test). During treatment the s.e. means of prolactin levels in the CB 154- treated rats were all less than $\pm$ 3 and are not shown in the diagram. A cyclic pattern of vaginal smears was displayed by all individuals in both groups before and during treatment. Due to the occasional 5- instead of 4-day cycle the oestrous cycles of certain individuals were out of phase towards the end of treatment and this may account for the less pronounced cyclic variations in plasma prolactin in the control group.

Figure 18. Effect of daily administration of CB 154 on plasma prolactin levels



At dioestrus (day 0) rats were allocated to groups (n = 6 in each) to receive daily s.c. injections of either CB 154 (5mg/kg body weight) or corn oil (1ml/kg body weight). Vertical lines indicate s.e. means. The s.e. means during CB 154 treatment were all less than 3ng/ml and are not shown.

When these results are compared with those in Fig. 17 it would appear that under the experimental conditions described lower plasma prolactin levels were obtained in CB 154-treated rats than in ovariectomised rats.

The plasma prolactin levels in 3 rats given a single dose of CB 154 are compared with those of 6 control animals given only corn oil vehicle during the 24 hour period immediately following injection in Fig. 19. There was no difference between the prolactin levels of the 2 groups up to 2 hours after the injection. However, after 4 and 24 hours the mean plasma prolactin levels in CB 154- treated rats were significantly lower than those of control rats ($p < 0.05$ and $p < 0.025$ respectively by Student t-test).

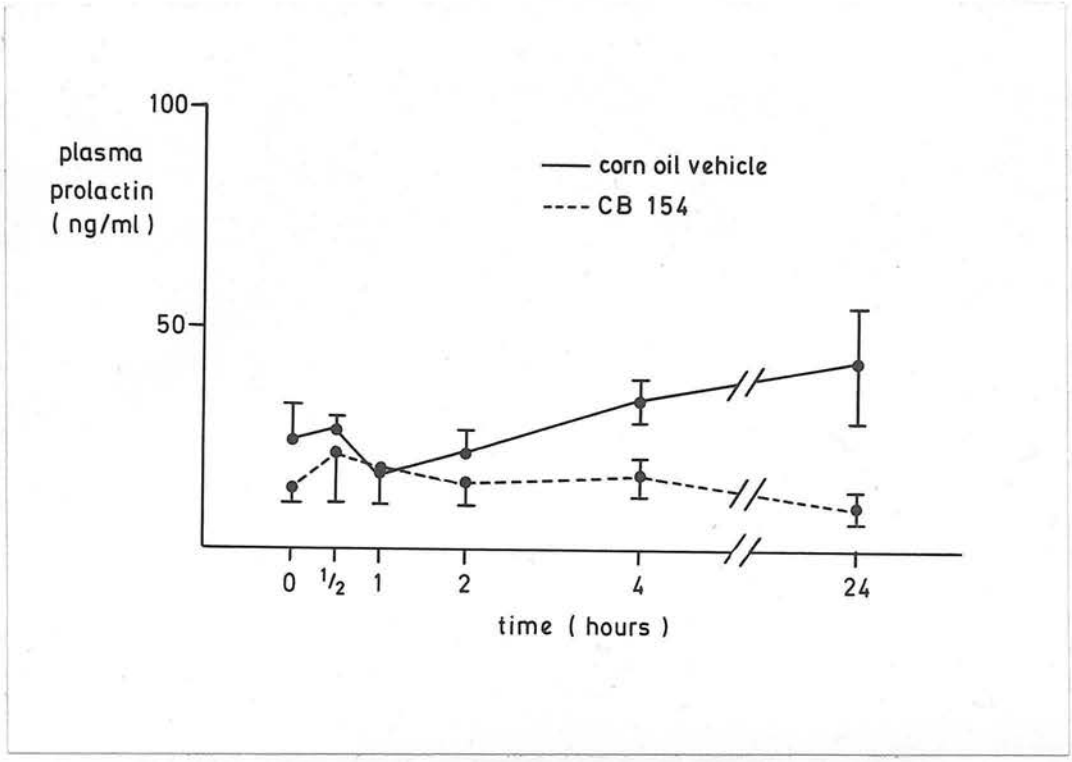
4. Perphenazine and CB 154 on plasma oestradiol levels

The minimum volume of plasma on which oestradiol levels in the female rat could be reliably determined by radioimmunoassay was 2.5ml. This was only routinely available at sacrifice when blood was collected by aortic exsanguination. However, oestradiol levels vary considerably throughout the oestrous cycle in the rat (Yoshinaga, Hawkins & Stocker, 1969; Butcher, Collins & Fugo, 1974) and a single estimation of oestradiol at sacrifice may therefore be unrepresentative in animals which continue to cycle during the treatment period.

In order to determine the overall effect of treatment on oestradiol levels in rats from the intact treatment groups it was decided to take a series of daily tail vein samples and bulk the plasma of individual animals for measurement of oestradiol. This would give an indication of the average oestradiol level over a given period which could then be compared with the level in plasma from the same animal at sacrifice. A further comparison could also be made with both sets of values between the different treatment groups.

The effects of perphenazine and CB 154 administration (5mg drug/kg body weight daily) on plasma oestradiol levels were therefore studied using the following protocol. Rats with regular oestrous cycles received 12 daily injections of drug or corn oil vehicle commencing on the day of dioestrus (day 0).

Figure 19. Plasma prolactin levels after a single dose of CB 154



At noon of dioestrus (time = 0) rats received a single s.c. injection of either (a) CB 154, 5 mg/kg body weight (n = 3), or (b) corn oil vehicle, 1ml/kg body weight (n = 6). Vertical lines indicate s.e. means.

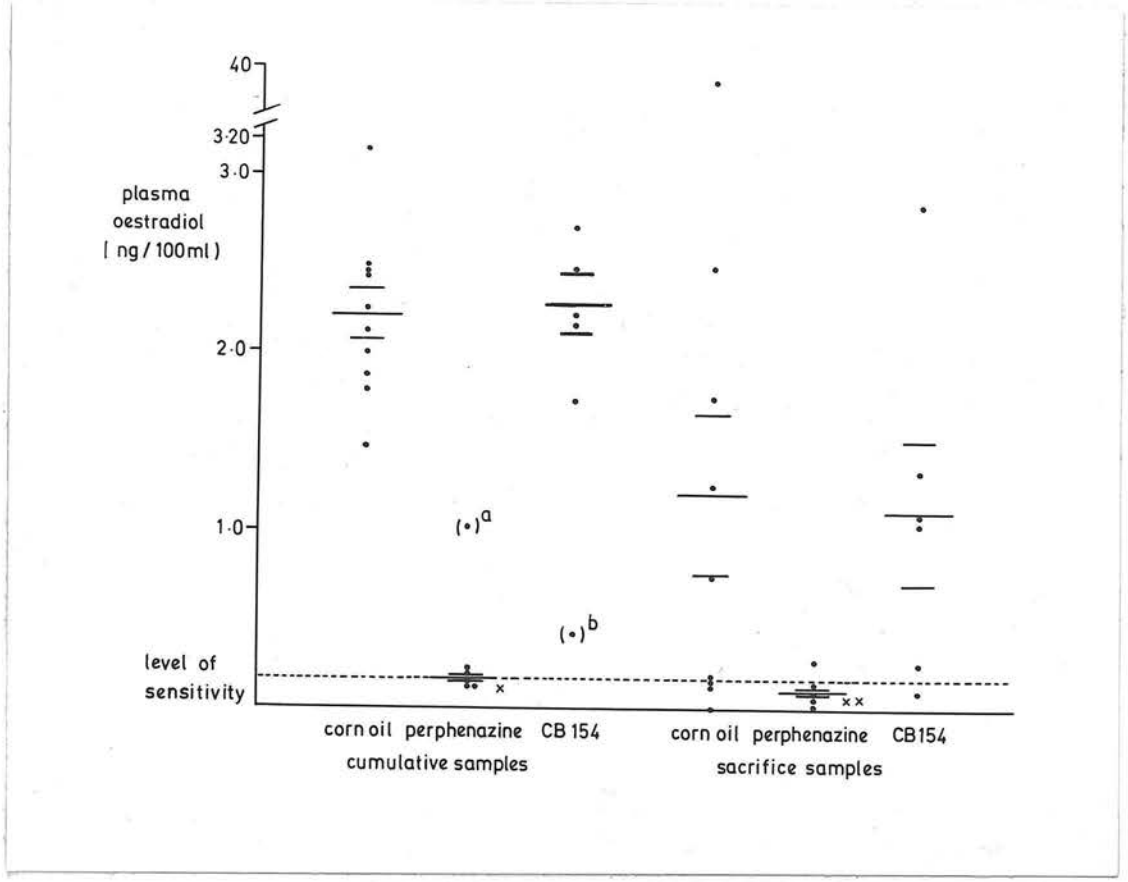
On each of the last 8 days of treatment (days 5 - 12) 1ml of blood was taken from the tail vein between 1430 and 1500 hours, immediately prior to the daily injection, or, on day 12, immediately prior to sacrifice. All animals were sacrificed 24 hours after the 12th injection, irrespective of the stage of cycle. Aliquots of plasma (400µl) from the 8 daily blood samples for each animal were pooled to give a final volume of 3.2ml (cumulative sample). 3ml of this sample was assayed for oestradiol along with a similar volume of plasma collected by aortic exsanguination at sacrifice. Plasma oestradiol levels in individual cumulative and sacrifice samples are listed in Table 9 and diagrammatically shown in Fig. 20.

All animals in the corn oil- and CB 154-treated groups continued to cycle regularly except for one of the CB 154-treated rats, 288L (cumulative value indicated by $(\ )^b$ in Table 9 and Fig. 20) which displayed a continuous dioestrus-type smear throughout the sampling period. Amongst the cycling rats a strict 4-day cycle was not always adhered to so that not all rats were in dioestrus at sacrifice on day 12. However, all cycling animals appeared to pass through proestrus twice during the 8 day sampling period. In the perphenazine-treated group all animals displayed a constant dioestrus-type smear pattern throughout the sampling period, apart from one individual, 125L (cumulative value indicated by $(\ )^a$ in Table 9 and Fig. 20) which appeared to undergo one cycle in the first half of the 8-day sampling period.

The range of oestradiol levels in the cumulative samples of both the cycling control and CB 154-treated rats was relatively small and the mean values from both these groups were very similar. The oestradiol level in the cumulative sample of the CB 154-treated animal $(\ )^b$ which did not appear to cycle was much lower than those of the other members of the group.

The most striking observation was that of the extremely low levels of oestradiol measured in the cumulative samples of the perphenazine-treated rats whose cycle appeared to be arrested in dioestrus. The values in this group

Figure 20. Effect of perphenazine and CB 154 on plasma oestradiol levels



Rats received daily s.c. injections of drug (5mg/kg body weight) or corn oil vehicle (1ml/kg body weight) for 12 days. A cumulative plasma sample of 3.2ml was obtained for each rat by pooling 400µl aliquots of plasma obtained from blood samples removed daily from the tail vein on days 5 - 12 of treatment.

$(\ )^a$ and $(\ )^b$ were not included in the determination of means and s.e. means (horizontal bars) - see text.

^{x} and ^{xx} = significantly lower than the control group by the Student's t-test, $p < 0.0005$ and < 0.05 respectively.

Table 9

Effect of perphenazine and CB 154 on plasma oestradiol in intact rats

Treatment	Rat no.	Plasma oestradiol (ng/100 ml) Cumulative sample	Sacrifice sample
Corn oil	153L	1.78	0.01
	158L	2.00	1.75
	176L	2.45	0.74
	178L	2.12	-
	184L	1.57	1.25
	186L	2.41	0.1
	306L	2.24	0.18
	311L	1.87	0.16
	316L	2.48	4.14
	329L	3.14	2.49
	mean	2.21	1.21
	s.e. mean	0.14	0.45
Perphenazine	125L	(1.02)[a]	0.08
	129L	0.20	0.04
	149L	0.24	0.04
	165L	0.12	0.00
	171L	0.12	0.13
	181L	0.15	0.28
	mean	0.17	0.09
	s.e. mean	0.02	0.04

Table 9 (continued)

Treatment	Rat no.	Plasma oestradiol (ng/100 ml) Cumulative sample	Sacrifice sample
CB 154	284L	1.73	2.84
	285L	2.16	1.33
	288L	(0.42)[b]	0.10
	312L	2.22	1.10
	315L	2.48	1.04
	327L	2.71	0.26
	mean	2.26	1.11
	s.e. mean	0.17	0.40

( )[a+b] - figures not included in statistical analysis because rats showed atypical vaginal smear patterns

- full details are given in text

showed no overlap with those of the control group and were therefore significantly different ($p < 0.0005$ by the Student t-test). Even allowing for the dilution effect of pooling aliquots of plasma, these extremely low levels imply that no single aliquot taken throughout the sampling period contained substantial quantities of oestradiol. Only the rat ( )[a] which appeared to undergo one oestrous cycle had an exceptional level of oestradiol compared to those of the others in the group.

In comparison to oestradiol levels in cumulative samples, sacrifice values for plasma oestradiol in the control and CB 154-treated groups have a wide range, presumably due to the different stages of cycle at which the animals were killed. The mean sacrifice levels of plasma oestradiol were lower than the cumulative means and again there was no significant difference between values from control and CB 154-treated groups. As would be expected from the values of cumulative samples, oestradiol levels at sacrifice in the perphenazine-treated rats were very low and significantly different from those of the control group ($p < 0.05$ by the Student t-test) although some values from control rats were also very low.

When the sacrifice values are compared with the cumulative values (Table 9), it can be seen that there is little correlation between the levels of oestradiol in the cumulative and sacrifice samples of individuals. Thus certain individuals in the control group with very low levels of oestradiol at sacrifice had relatively high values of oestradiol in their cumulative samples. However, since all animals with continuously low oestradiol levels throughout treatment have low sacrifice values (e.g. in perphenazine-treated group) and a higher mean level of oestradiol at sacrifice occurred in the groups of cycling rats with substantial oestradiol levels in the cumulative samples, inter-group comparisons of plasma oestradiol content at sacrifice are still valid.

Although this method of cumulative sampling consists of pooling aliquots taken only once every 24 hours, the oestradiol values in these samples correlate

remarkably well with the individual's cyclicity as measured by the vaginal smear pattern. This is evident not only from the lower oestradiol values obtained in the non-cycling perphenazine-treated rats, but also from the intermediate oestradiol level of the perphenazine-treated individual ( )[a] which appeared to cycle once and from the low oestradiol level of the CB 154-treated individual ( )[b] which remained in constant dioestrus.

5. Summary

Using the method of blood sampling from the tail vein under ether anaesthesia it has been possible to show that s.c. administration in corn oil at a daily dose of 5mg/kg body weight perphenazine elevated, and CB 154 depressed, plasma prolactin levels in intact rats. Whereas CB 154 does not appear to inhibit the ovulatory cycle in the rat, intact perphenazine-treated rats exhibit constant dioestrus-type smears and from the oestradiol levels obtained in cumulative samples it seems likely that oestradiol output is greatly inhibited by this drug. Plasma prolactin levels were also decreased in ovariectomised animals as compared to intact controls.

III. Effect of hormonal manipulation on tumour growth and testosterone metabolism by DMBA-induced mammary tumours

In order to study the relationships between plasma hormones, tumour growth and tumour steroid metabolism, rats bearing DMBA-induced mammary tumours were allocated to 5 treatment regimes designed to alter the levels of plasma hormones important in controlling the growth of these tumours. Incubation of the tumours with [^{3}H] testosterone after treatment provided a measurement of the capacity of tumour to convert testosterone to its major metabolites in vitro which could then be related to tumour growth and plasma levels of prolactin and oestradiol.

1. Experimental design

Only tumours which were actively growing and borne by rats showing regular oestrous cycles of 4 days were considered for this study. At the dioestrus stage of the cycle the rats were allocated to one of the 5 treatment regimes shown in Table 10. All animals were sacrificed when exhibiting a dioestrus-type vaginal smear, so that the vaginal smear appearance was uniform in all treatment groups at the start and completion of treatment (perphenazine-treated and ovariectomised animals displayed a constant dioestrus-type vaginal smear pattern). Occasionally the treatment period was extended beyond the intended 12 days to accommodate rats whose cycle had varied during treatment such that a dioestrus-type vaginal smear was not seen on day 12. It was also sometimes necessary to sacrifice animals with rapidly regressing tumours before the twelfth day of treatment so that sufficient tumour tissue was available for incubation.

At sacrifice tumours were excised and divided into portions for testosterone metabolism studies, histology and, when sufficient, determination of DNA content. Necrotic tumours and tumours which were not classified as adenocarcinomas were not used for the study.

Legend for Tables 10 - 15

The values obtained from groups 2, 3, 4 and 5 were compared to those of group 1 by variance analysis plus Dunnett's test and those of group 4 with those of group 5 by the F-test (- = not significant; > and >> = greater than, and < and << = less than, the control values at $p < 0.05$ and $p < 0.01$ levels respectively). The first symbol after group 5 values refers to the comparison with group 1 values and the lower symbol refers to the comparison with group 4 values. When the variances within groups were deemed incomparable by Hartley's test ($p < 0.01$), all data were transformed to natural logarithms (ln) before the variance analysis. Where this was done the ln means and corresponding standard deviations (s.d.) are given in the tables with the geometric means (= antilogarithms of the ln means) in brackets. The arithmetic means are given in the complete tables of results. Where additional analyses have been employed, a description is given with the appropriate table.

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Table 10

Treatment regimes

	Group	Endocrine status	Injection (s.c.)	Number of rats	Days since DMBA	Days of treatment
1.	IC	intact	corn oil	11	89.5	13.3
2.	I + P	intact	perphenazine	9	118.1>	14.9-
3.	I + B	intact	CB 154	7	97.9-	12.6-
4.	Ovx C	ovariectomised	corn oil	10	107.3-	9.6<
5.	Ovx + P	ovariectomised	perphenazine	7	93.3-	14.0->
		within group	pooled s.d.		21.8	1.9
		within group	d.f.		39	39

The complete results from the individual rats in each treatment group are given at the end of this section in Tables 18 - 22. Data pooled for the statistical analyses of intergroup variations are given in Tables 10 - 13 and 15, and are illustrated diagrammatically in Figs. 21 - 29. The interrelationships between certain parameters have also been considered on an individual basis by investigating correlations within groups and over all groups taken together.

Although allocation to a treatment group usually occurred as soon as a rat had a sufficiently large, growing tumour and was cycling regularly, there was a considerable range in times between carcinogen administration and allocation to a treatment group. Only when all data were compiled at the end of the study was it realised that average time between the administration of carcinogen and the allocation to treatment groups was significantly greater in the intact, perphenazine-treated group than in the intact control group (Table 10). The relevance of this situation is considered further in the section on individual intercorrelations.

2. Vaginal smear pattern

All intact rats given corn oil vehicle or CB 154 continued to cycle regularly throughout the treatment period. Ovariectomised animals, whether given corn oil or perphenazine, displayed a constant dioestrus-type vaginal smear pattern. Perphenazine administration to intact rats also produced a generalised pattern of persistent dioestrus smears but this effect was not always immediate, with some rats apparently undergoing one further cycle before arresting in dioestrus.

3. Plasma prolactin levels

Blood samples for the assay of plasma prolactin were removed from the tail vein between 1030 and 1200 hours immediately prior to starting treatment and at the same time of day at sacrifice, 24 hours after the final administration of drug or vehicle. The mean plasma prolactin values for the groups are shown in Table 11.

Table 11

Plasma prolactin levels in rats bearing tumours used for steroid metabolism studies

	Treatment group	Number of rats	Plasma prolactin (ln ng/ml + geom. mean) initial		Plasma prolactin (ln ng/ml + geom. mean) final	
1.	I C	11	3.06	(21.4)	2.92	(18.5)
2.	I + P	9	3.23	(25.3)-	4.23	(68.4)>>
3.	I + B	7	3.26	(26.0)-	1.64	(5.2)<<
4.	Ovx C	10	3.15	(23.3)-	1.78	(5.9)<<
5.	OVX + P	7	3.32	(27.7)-	4.42	(83.1)>>
	within group	pooled s.d.	0.95		0.61	
	within group	d.f.	39		39	

Legend on page R-51.

Blood samples for plasma prolactin assay were taken immediately prior to the start of treatment (initial), and at sacrifice - 24 hours after the last injection (final).

Pretreatment values were similar in all 5 groups. In intact animals the final prolactin levels showed no difference from pretreatment values after corn oil treatment, but were significantly raised by perphenazine and depressed by CB 154. In ovariectomised rats given corn oil the final plasma prolactin levels were lower than pretreatment values whereas an elevation from pretreatment to final levels was observed in ovariectomised rats given perphenazine. These differences were all significant by the paired t-test ($p < 0.05$). As pretreatment values were similar in all groups this meant that in perphenazine-treated rats (intact and ovariectomised), sacrifice levels were significantly higher and in ovariectomised rats given corn oil and in CB 154-treated rats sacrifice levels were significantly lower than the plasma prolactin levels in the control group at sacrifice.

The prolactin values obtained at sacrifice are similar to those reported in the previous section where more detailed studies were carried out on non-tumour-bearing rats. Since the single sacrifice samples tend to reflect the plasma prolactin levels throughout the treatment period the various treatments applied to the tumour-bearing rats in this study appear to have had the desired effect on plasma prolactin.

4. Plasma oestradiol levels

Plasma oestradiol concentration was measured in a large volume of blood (8-12ml), withdrawn from the abdominal aorta at sacrifice. The mean values obtained from each group are shown in Table 12. Mean plasma oestradiol levels in both groups of ovariectomised animals were obviously lower than those of the intact control group and since they were around the limits of detection of the assay for the volume of plasma available, were not statistically analysed.

The mean oestradiol level in the perphenazine-treated intact group was almost half that of the control group, and although the difference was not significant by variance analysis by the Wilcoxon rank test the difference was significant at the 5% level. Plasma oestradiol levels were however significantly

Table 12

Plasma oestradiol levels in rats bearing tumours used for steroid metabolism studies

	Treatment group	Number of rats	Plasma oestradiol (ng/100ml)
1.	I C	11	0.68
2.	I + P	9	0.37- *
3.	I + B	7	1.17>
	within group	pooled s.d.	0.43
		d.f.	24
4.	Ovx C	10	0.12
5.	Ovx + P	7	0.21

Legend on page R-51.

Blood samples for plasma oestradiol assay were taken at sacrifice - 24 hours after the final injection. Limit of detection of the oestradiol assay - 0.16ng/ml.

* The difference between the intact + perphenazine and the intact control group is significant when tested by the Wilcoxon rank test ($p < 0.05$).

higher in the CB 154-treated group than in the control group by variance analysis.

These results from the intact groups are not in complete accord with the data presented for non-tumour-bearing intact rats in the previous section. In that study plasma oestradiol levels at sacrifice were clearly significantly lower in the perphenazine-treated group compared to the control group whereas there was no difference between the values of the CB 154-treated animals compared to those of the control group. The apparent dissimilarity between the 2 sets of results can be accounted for by the difference in oestradiol sacrifice values in the control groups of the 2 studies. The control group in the previous section had a mean sacrifice value of 1.21ng/100ml compared with 0.68ng/100ml for the control group in this study. This difference may be due to the fact that all animals in the present study were sacrificed in dioestrus whereas in the earlier study the date of sacrifice was determined by a fixed number of days of treatment and not by stage of the cycle. An alternative explanation may be that the time of sampling was earlier in the present study.

Whilst it was demonstrated in the cumulative sampling study that treatment with perphenazine clearly suppressed plasma oestradiol levels in intact rats, and that CB 154 had no apparent effect on oestradiol levels over the treatment period, it would appear from the results of the present study that the plasma oestradiol levels at dioestrus may be higher in CB 154-treated rats than in control rats at the same stage of the cycle.

5. Tumour growth

Tumour size, expressed as a multiple of major and minor diameters, was measured every 2 or 3 days before and during treatment. The individual tumour sizes and growth rates at the start and end of treatment are given in Tables 18 - 22. The growth rates were derived from the change in tumour size over the week preceding the start of treatment and over the last week of treatment. Since the tumour growth rate in certain rats varied throughout the treatment

period, only the measurements taken over the last week were used to assess tumour growth so that as nearly as possible the actual growth rates at the time of sacrifice were expressed. Therefore, the final growth rate does not necessarily correspond to the difference in tumour size at the start and at the end of the treatment period.

The mean values of tumour size and growth rate are compared in Table 13, and the mean tumour growth rates are shown in Figs. 21 and 22. For direct comparison of growth rates in the figures individual tumour size was first expressed as a percentage of that on the day treatment commenced before calculating the mean at each time point. Individual examples are given in Figs. 23 and 24.

It can be seen from Table 13 that both the initial size and initial growth rate of tumours in CB 154-treated and ovariectomised-plus-perphenazine-treated groups are significantly higher than those of the control group. The difference in initial tumour size resulted from the deliberate allocation of rats with larger tumours to the 2 groups where tumour regression was expected so that at sacrifice sufficient tumour mass was available for steroid metabolism studies. Conversely , if rats with large tumours were allocated to the intact-plus-perphenazine treatment group in which rapid tumour growth was expected, there was an increased risk of complications such as tumour necrosis before the end of treatment. The higher tumour growth rate observed in the groups with the greater initial tumour size may reflect a general relationship between tumour growth rate and tumour size due to the way in which these parameters were expressed. When tumour size was expressed as a percentage of the size at the start of treatment (Figs. 21 and 22), there was no apparent initial difference in growth rate. In general, tumours in the intact control group given corn oil grew at a similar rate before and during treatment. One of the tumours did cease to grow during treatment.

Table 13

Size and growth rate of tumours

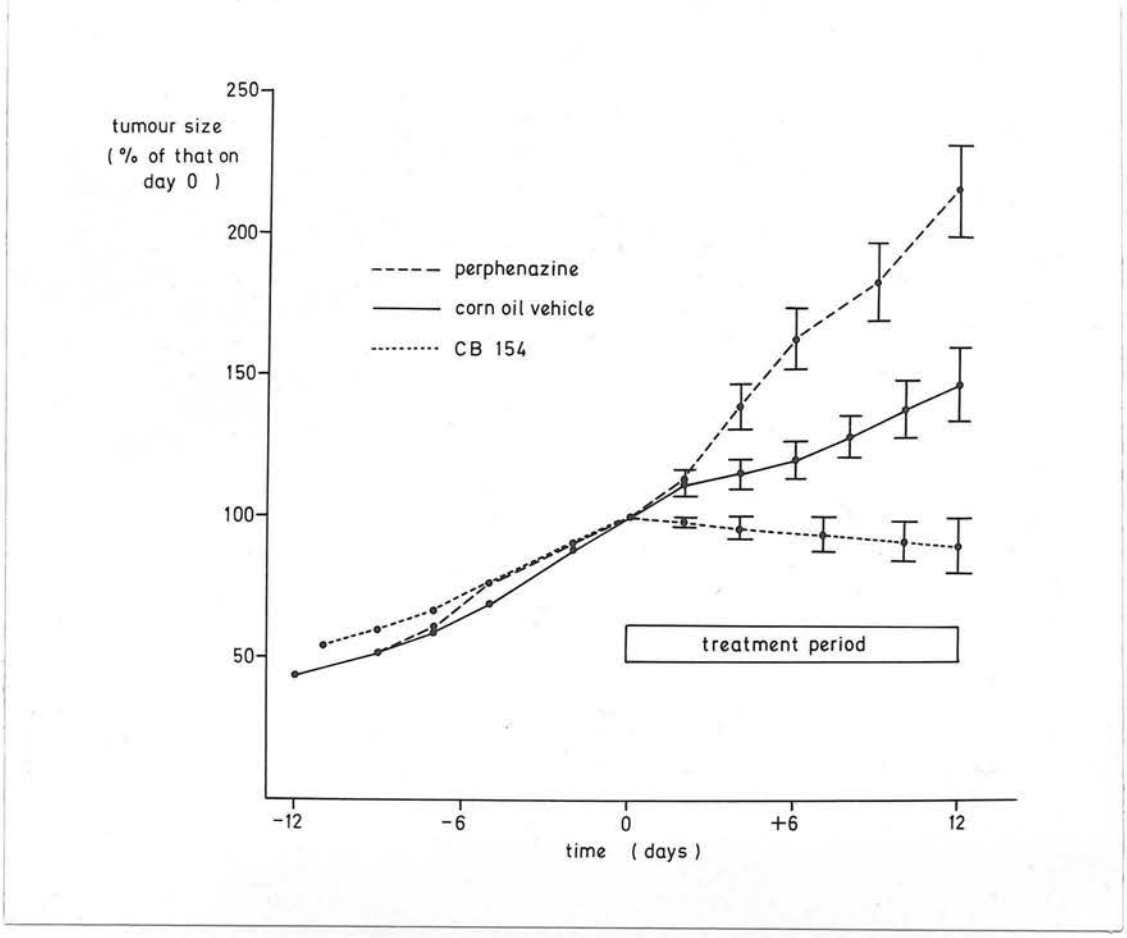
used for steroid metabolism studies

	Treatment group	Number of tumours	Tumour size (ln cm^2 + geom. mean) initial		Tumour size (ln cm^2 + geom. mean) final		Tumour growth rate (cm^2/week) initial	Tumour growth rate (cm^2/week) final
1.	I C	14	0.92	(2.52)	1.30	(3.67)	0.89	0.68
2.	I + P	10	0.68	(1.98)-	1.57	(4.81)-	0.70-	1.39-*
3.	I + B	11	1.64	(5.13)>>	1.46	(4.30)-	1.70>	-0.29<
4.	Ovx C	10	1.89	(6.92)>>	1.31	(3.69)-	1.59>	-2.41<<
5.	Ovx + P	10	1.00	(2.71)-<<	1.15	(3.16)- -	1.01- -	-0.16< >>
	within group	pooled s.d.	0.39		0.47		0.68	0.87
	within group	d.f.	50		50		50	50

Legend on page R-51

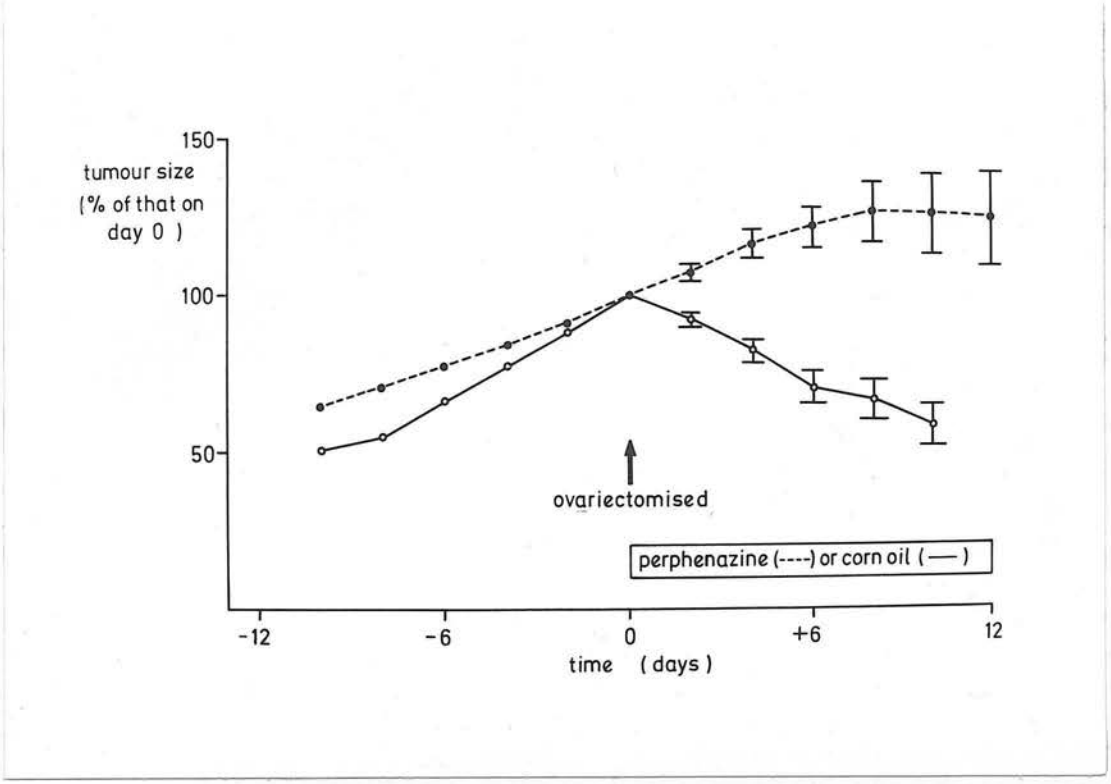
* Significantly higher than the control value when compared by the Wilcoxon rank test ($p < 0.05$).

Figure 21. Effect of perphenazine and CB 154 on the growth of DMBA-induced tumours in intact rats



Rats received daily s.c. injections of perphenazine or CB 154 (both at 5mg/kg body weight) or corn oil vehicle (1ml/kg body weight) starting on day 0. Tumour sizes were calculated individually as percentages of that on day 0 and the means of these percentages at given times were used to construct the graph. Vertical lines indicate s.e. means.

Figure 22. Effect of perphenazine on the growth of DMBA-induced mammary tumours after ovariectomy



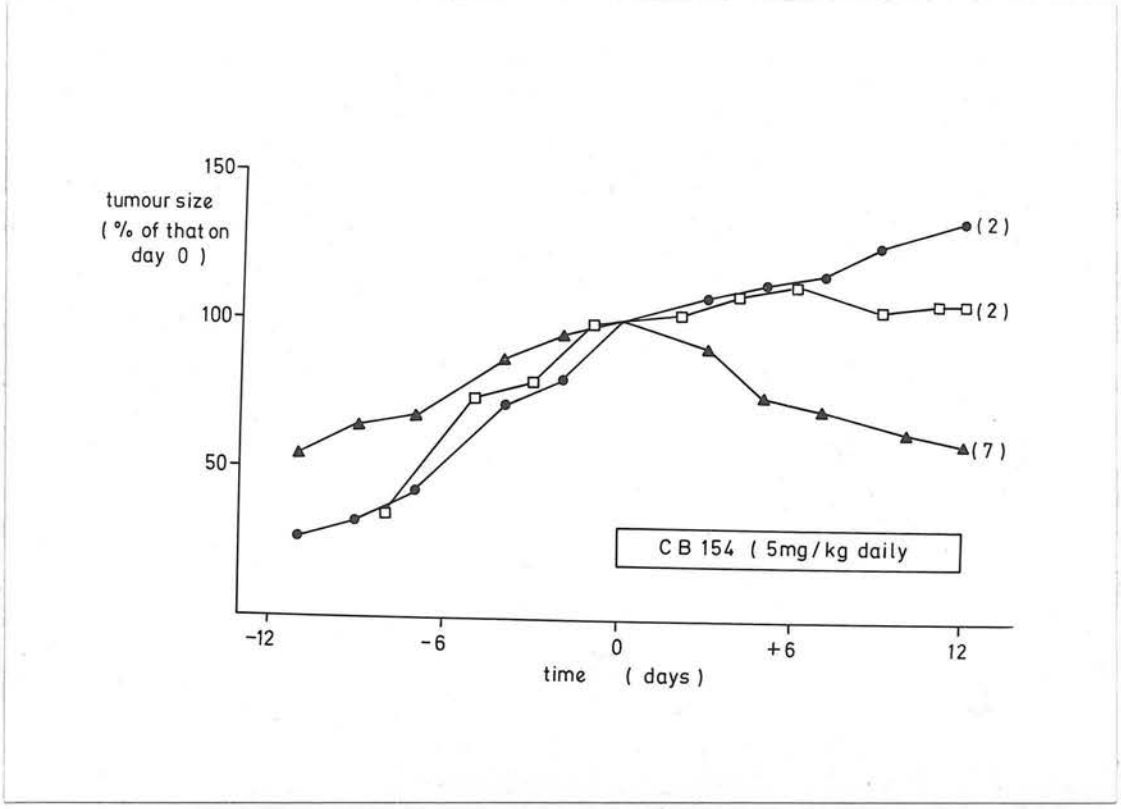
The experimental design and expression of results were the same as described in Figure 21, except that all rats were bilaterally ovariectomised on day 0. Vertical lines indicate s.e. means.

Mean tumour growth rate in intact animals was accelerated by the administration of perphenazine. All tumours in this group continued to grow throughout the treatment period, and in all but 2 of the 10 tumours the growth rate was increased. The percentage increase in tumour size and the final tumour growth rate of the intact perphenazine-treated rats were significantly higher than corresponding values for the intact control group by the Wilcoxon rank test ($p < 0.05$).

The mean tumour growth pattern of intact rats treated with CB 154 became static after the start of treatment. The result was that by the end of treatment the percentage change in tumour size and final tumour growth rate was significantly less in the CB 154-treated animals than in the control group. Expressing the results as means does however obscure the responses of individual tumours. Typical examples of the 3 main types of tumour growth with the number of tumours classified into each type are shown in Fig. 23. Of the 11 tumours in this group, 7 regressed, 2 continued to grow and in the remaining 2 growth was checked, but resumed by the end of treatment.

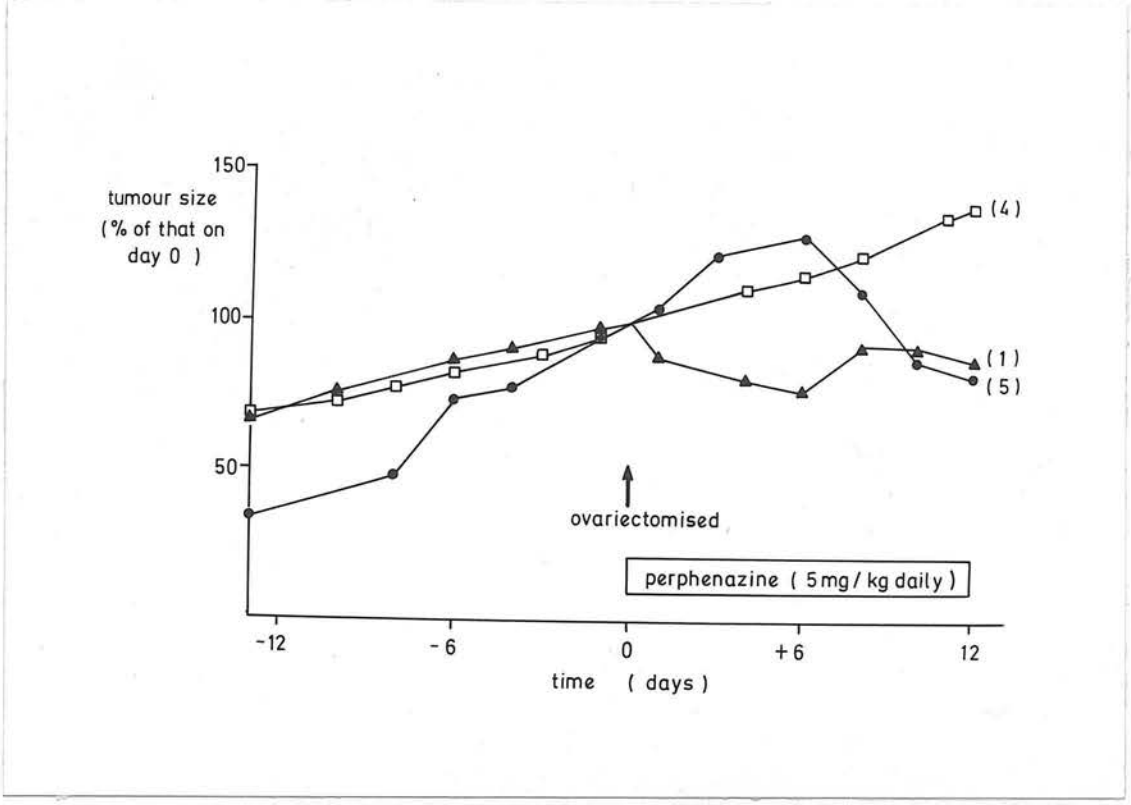
The mean tumour growth rates of animals in the 2 ovariectomised groups are shown in Fig. 22. In the animals which were ovariectomised and received corn oil all tumours showed immediate regression which continued throughout the treatment period. The administration of perphenazine from the time of ovariectomy prevented immediate tumour regression and the mean change in tumour size over the treatment period and the final growth rate were significantly different between the 2 ovariectomised groups. There was however a large variation in the growth pattern of tumours in ovariectomised rats given perphenazine. Individual examples of 3 broad categories of tumour growth with the number of tumours which fit into these categories are given in Fig. 24. Of the 10 tumours in this group, 5 regressed after an initial growth period lasting 5-10 days, one showed little net change in size and 4 grew throughout the treatment period.

Figure 23. Growth patterns of individual DMBA-induced mammary tumours in intact rats given CB 154



The differing growth patterns of 3 tumours in CB 154-treated rats are displayed. From the 11 tumours incubated for steroid metabolism studies in this group, the number typified by a particular growth pattern is given in brackets.

Figure 24. Growth patterns of individual DMBA-induced mammary tumours in ovariectomised rats given perphenazine



The differing growth patterns of 3 tumours in rats receiving perphenazine after ovariectomy are displayed. From the 10 tumours incubated for steroid metabolism studies in this group, the number typified by a particular growth pattern is given in brackets.

The variation in tumour growth seen in certain groups could have been due to the variations in the extent of hormonal changes caused by the various treatment regimes. However, it seems unlikely that this is the full explanation since variations in tumour growth were often seen within one rat bearing 2 or more tumours (Table 22). Thus to a certain degree variations of the tumour growth in response to particular treatment would seem to depend on the properties of the individual tumours.

6. Metabolism of testosterone by tumours

At sacrifice tumours were excised and an accurately weighed portion of 0.5g was homogenised and then incubated with 50 x 10^6 d.p.m. of [7α-^{3}H] testosterone in 7.5ml Krebs-Ringer phosphate buffer, pH 7.4, for one hour at 37°C in the presence of an NADPH generating system as described in full in the METHODS section. Total testosterone metabolism and the net formation of 5α-dihydrotestosterone and 5α-androstanediol were determined by measuring the percentage incorporation of radioactive label into the individual steroids following their purification and characterisation. Table 14 gives representative examples of the characterisation of precursor and metabolites following the incubation of a tumour from each of the 5 groups. Data for testosterone metabolism by the individual tumours are given in Tables 18 - 22 and are pooled for inter-group statistical analysis in Table 15. Total 5α-reduction has been expressed as the sum of the percentages of 5α-dihydrotestosterone and 5α-androstanediol production. The ratio of 5α-androstanediol over 5α-reduction has also been calculated to give an indication of the relative amount of 5α-reduced steroid which has further undergone hydroxylation at the 3-position.

All tumours investigated under the conditions described metabolised testosterone to 5α-dihydrotestosterone and 5α-androstanediol and in every case the quantity of 5α-androstanediol recovered was greater than that of 5α-dihydrotestosterone, in a ratio of 4:1 on average. In all but 4 of the 55 tumours

Table 14

Characterisation of steroids in representative tumour incubates from each of the five groups studied

Rat and tumour (group)	Carrier steroid	Derivative	Final solvent system	S.A. (d.p.m./nmol)	% of total d.p.m.
332E RIn	(I C)				
		testo free	III	13,605	
	testosterone	testo acetate	IV	13,256	47.75
		Δ^4-dione	III	13,506	
		5α-DHT free	III	2,942	
	5α-DHT	5α-DHT acetate	IV	3,130	10.56
		5α-Adiol(3β)	III	2,916	
	5α-Adiol (3α)	5α-Adione	II	7,368	26.39
		5α-Adiol(3β)	III	7,710	
65H LNk	(I + P)				
		testo free	III	9,791	
	testosterone	testo acetate	IV	9,772	33.79
		Δ^4-dione	III	9,531	
		5α-DHT free	III	5,849	
	5α-DHT	5α-DHT acetate	IV	5,881	20.29
		5α-Adiol(3β)	III	5,863	
	5α-Adiol(3α)	5α-Adione	II	12,786	43.79
		5α-Adiol(3β)	III	12,699	

Table 14 (continued)

Rat and tumour (group)	Carrier steroid	Derivative	Final solvent system	S.A. (d.p.m./nmol)	% of total d.p.m.
354L RIn	(I + B)				
		testo free	III	10,897	
	testosterone	testo acetate	IV	10,574	39.53
		Δ^4-dione	III	10,736	
		5α-DHT free	III	1,231	
	5α-DHT	5α-Adiol (3β)	III	1,255	4.59
		5α-Adione	II	1,278	
	5α-Adiol (3α)	5α-Adione	II	11,694	42.79
		5α-Adiol (3β)	III	11,874	
48L LIn	(Ovx C)				
		testo free	III	21,499	
	testosterone	testo acetate	IV	21,855	77.00
		Δ^4-dione	III	21,211	
		5α-DHT free	III	1,135	
	5α-DHT	5α-Adiol (3β)	III	1,136	4.01
		5α-Adione	II	1,113	
	5α-Adiol (3α)	5α-Adione	II	3,982	13.68
		5α-Adiol (3β)	III	3,775	

Table 14 (continued)

Rat and tumour (group)	Carrier steroid	Derivative	Final solvent system	S.A. (d.p.m./nmol)	% of total d.p.m.
297K RAx	(Ovx + P)				
		testo free	III	8,903	
	testosterone	testo acetate	IV	8,969	37.51
		Δ^4-dione	III	8,988	
		5α-DHT free	III	1,367	
	5α-DHT	5α-Adiol (3β)	III	1,338	5.54
		5 -Adione	II	1,287	
	5α-Adiol (3α)	5α-Adione	II	7,753	32.76
		5α-Adiol (3β)	III	8,104	

conversion to the 5α-reduced products, 5α-dihydrotestosterone and 5α-androstanediol accounted for the majority of the testosterone metabolism. Although these generalisations apply to all groups certain quantitative differences were seen between the groups when the results were subjected to statistical analysis (Table 15).

Total testosterone metabolism (Fig. 25) by tumours from intact rats given corn oil ranged from 35.60 - 66.88% with a mean of 53.74%. Tumours from intact rats given perphenazine metabolised testosterone to a significantly greater extent with only one tumour metabolising less than 50% of the precursor. The mean level of testosterone metabolism by tumours from intact rats given CB 154 was similar to that of the control group but the range of values was remarkably small (47.91 - 63.15%). In contrast a large variation in the ability to metabolise testosterone was observed in both groups of ovariectomised animals. Although mean level of testosterone metabolism by tumours from ovariectomised rats given perphenazine was significantly higher than that of the ovariectomised control group, it was not significantly different from that of the control and numerically less than that of intact rats given perphenazine. The metabolism of testosterone by tumours from the ovariectomised control group was significantly lower than that found in all other groups.

The quantities of 5α-dihydrotestosterone recovered at the end of tumour incubation are shown in Fig. 26. Since 5α-dihydrotestosterone can be further converted to 5α-androstanediol during incubation, the quantity of 5α-dihydrotestosterone recovered reflects its total formation minus its further metabolism. This net formation of 5α-dihydrotestosterone by tumours from intact rats given perphenazine was significantly higher than by those of the intact control group. None of the other treatment regimes appeared to significantly change the net formation of 5α-dihydrotestosterone.

The production of 5α-androstanediol by the same groups of tumours is shown in Fig. 27. The formation of this metabolite was significantly higher by

Table 15

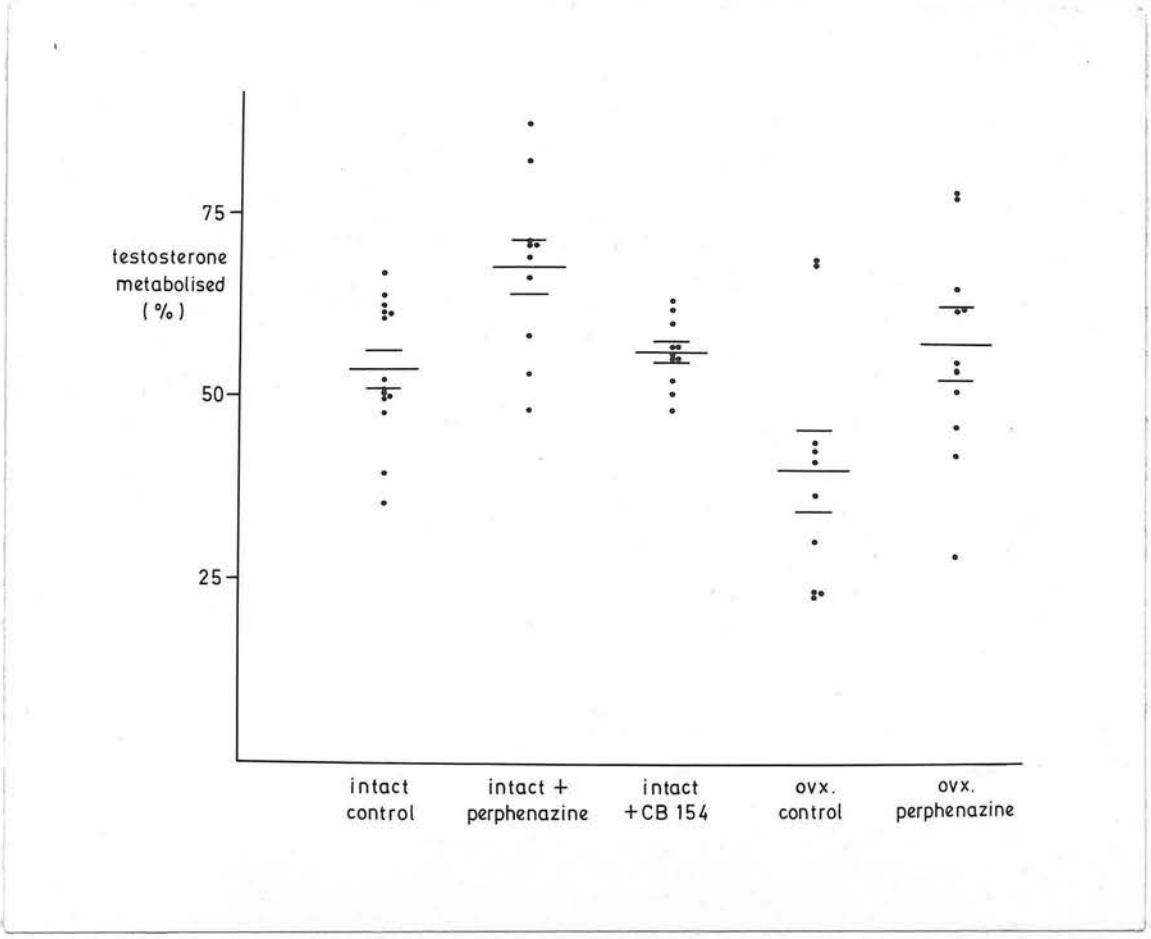
Metabolism of testosterone by tumours from the treatment groups studied

	Treatment group	Number of tumours	total metab.	Testosterone metabolism (%) 5α-DHT*	5α-Adiol	5α-red'n	5αAdiol / 5α-red'n
1.	I C	14	53.74	1.77 (5.88)	32.09	38.46	0.83
2.	I + P	10	67.65 >	2.59 (13.32) >	44.66 >	61.94 ≫	0.74 -
3.	I + B	11	56.07 -	1.90 (6.68) -	37.29 -	45.14 -	0.83 -
4.	Ovx C	10	40.11 <	1.41 (4.10) -	22.96 -	27.49 -	0.82 -
5.	Ovx + P	10	57.64 - ≫	1.81 (6.76) - -	33.22 - >	42.17 - >	0.81 - -
	within group	pooled s.d.	12.17	0.63	10.09	13.43	0.09
	within group	d.f.	50	50	50	50	50
Additional comparisons by the Student-Newman-Keuls test							
Group 2 vs 5			-	>	>	≫	-
Group 3 vs 4			>	-	>	>	-

Legend on page R-51

* data for the conversion of testosterone to 5α-DHT were transformed to natural logarithms.

Figure 25. Testosterone metabolism by DMBA-induced mammary tumours in rats of different hormonal status, (a) Total metabolism of testosterone



Treatment regimes are described in Table 10 and details of incubation procedures are given in the METHODS section. Each point represents the value from an individual tumour. Horizontal bars indicate the means and s.e. means for each group. Statistical analyses are given in Table 15.

Figure 26. Testosterone metabolism by DMBA-induced mammary tumours in rats of different hormonal status, (b) Conversion to 5α-dihydrotestosterone

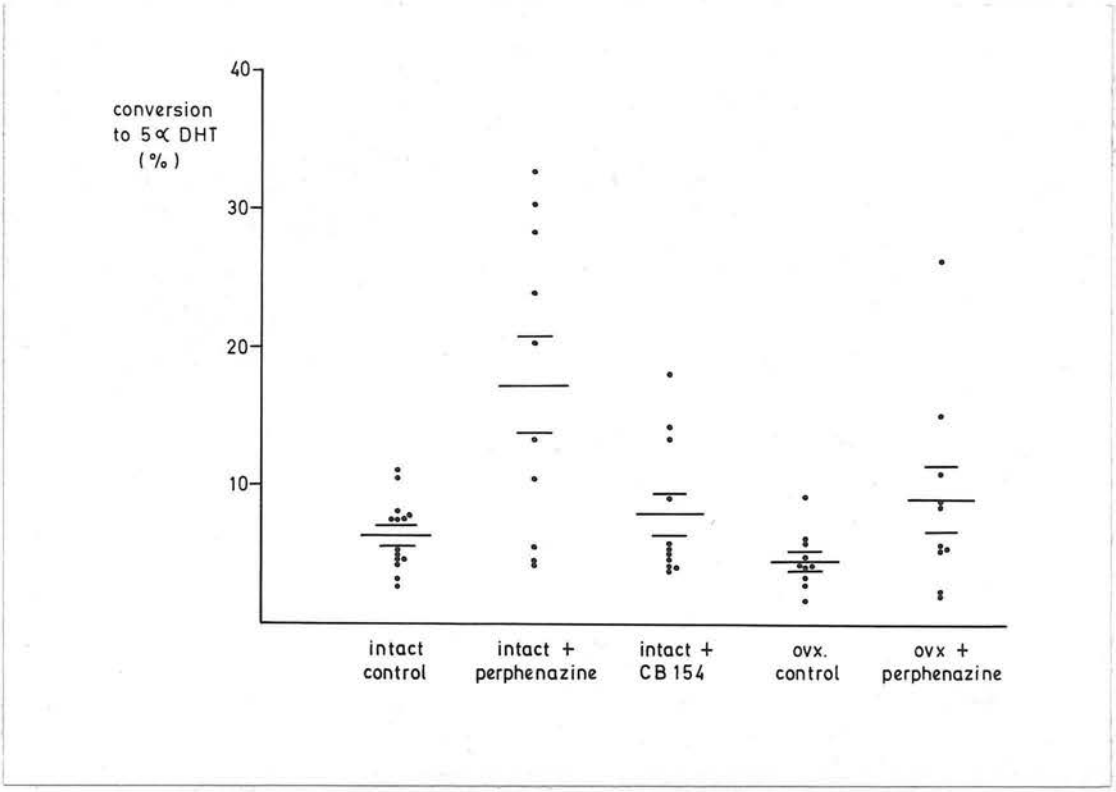


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Figure 27. Testosterone metabolism by DMBA-induced mammary tumours in rats of different hormonal status, (c) Conversion to 5α-androstanediol

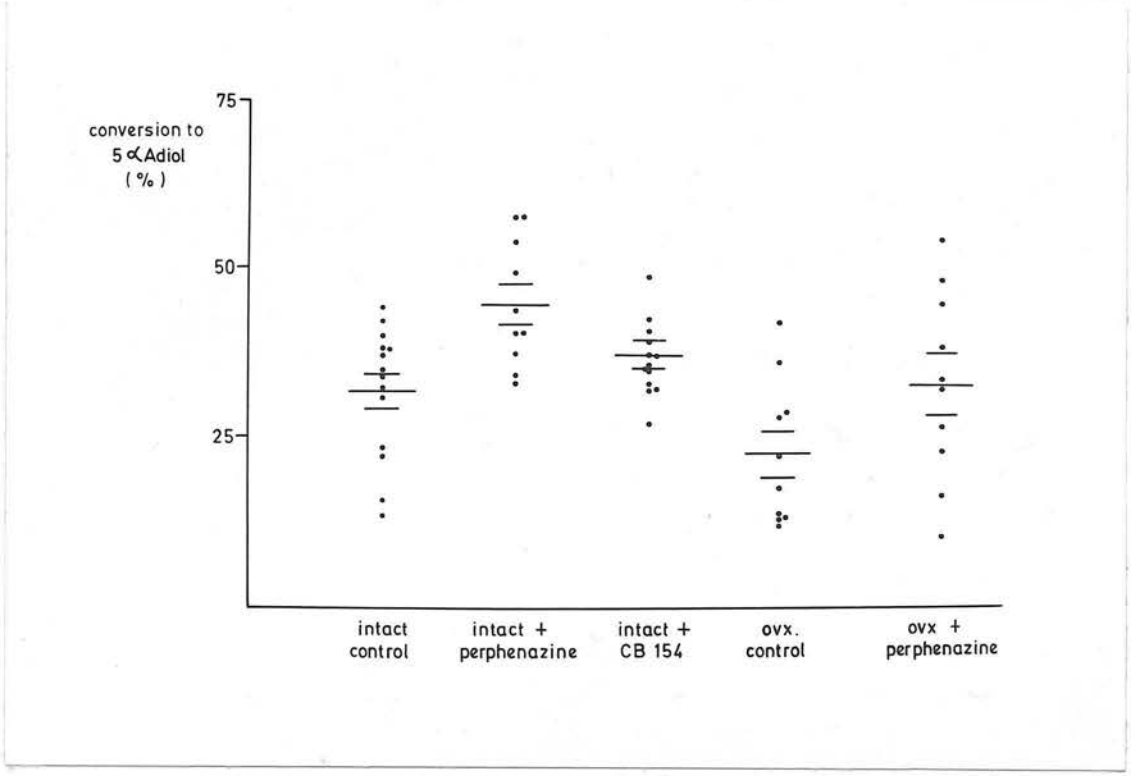

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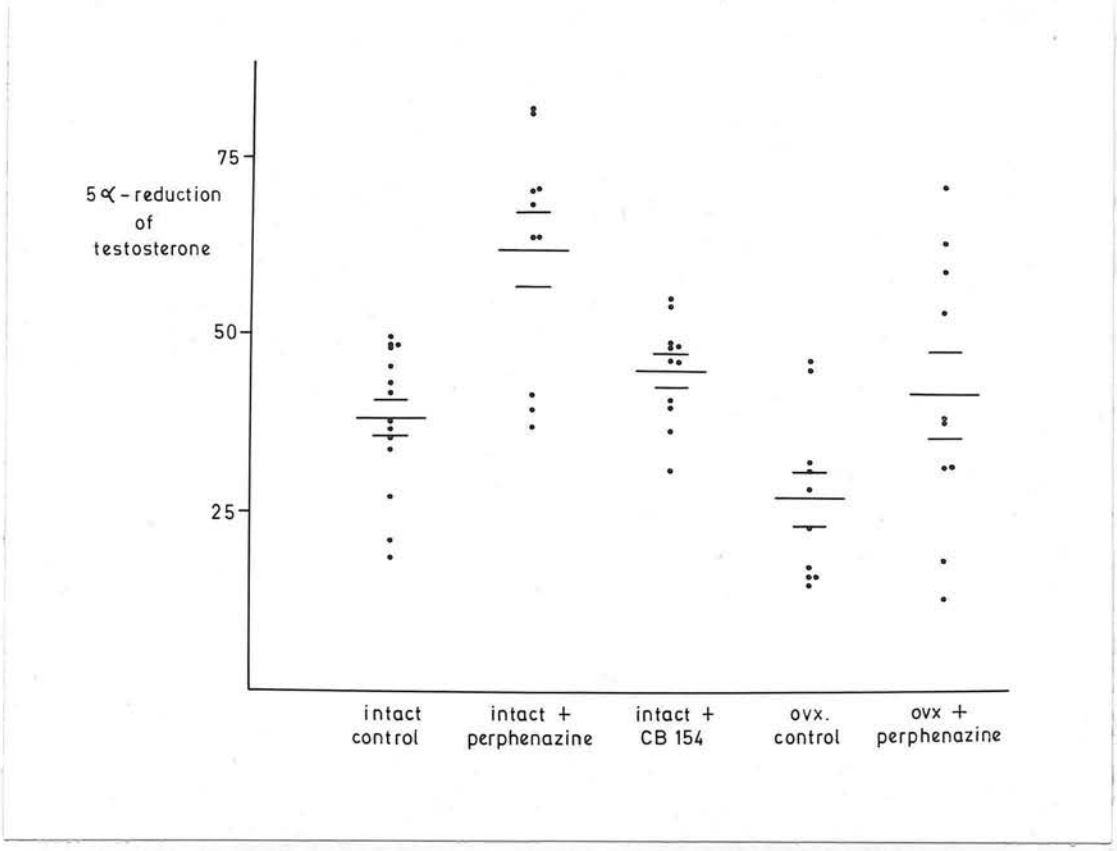
Figure 28. Testosterone metabolism by DMBA-induced mammary tumours in rats of different hormonal status, (d) 5α-reduction of testosterone



See legend of Figure 25

tumours from both intact and ovariectomised rats given perphenazine than by those of their respective control groups. However, the formation of 5α-androstanediol by tumours from the ovariectomised group given perphenazine was similar to that of the intact control group and significantly less than that of the intact group given perphenazine. The formation of 5α-androstanediol by tumours from intact rats given CB 154 did not differ significantly from that of the intact control group, but was significantly greater than that of the ovariectomised control group.

Tumour 5α-reduction of testosterone is shown in Fig. 28. The pattern of differences is similar to that described above for the production of the major component, 5α-androstanediol. In both intact and ovariectomised rats perphenazine treatment significantly stimulated the 5α-reduction of testosterone by DMBA-induced tumours. The effect was highly significant in the intact animals ($p < 0.01$), reflecting the individually significant elevations in the formation of both 5α-reduced metabolites. This increase in 5α-reduction is alone sufficient to account for the increased levels of testosterone metabolism observed in tumours from perphenazine-treated rats. The extent of the 5α-reduction of testosterone by tumours from the ovariectomised animals given perphenazine was similar to that found in the control group and significantly lower than that of the intact animals given perphenazine. 5α-reduction by tumours from intact rats given CB 154 did not differ significantly from that of the control group, but was significantly higher than that of the ovariectomised group given corn oil.

The mean ratio of 5α-androstanediol formation over 5α-reduction lay between 0.81 and 0.83 for all groups except the intact, perphenazine-treated group which gave a ratio of 0.74. The lower values in this group resulted from the greater contribution of 5α-dihydrotestosterone levels to 5α-reduction by some tumours in the group. However, the difference was not significant.

In summary, the administration of perphenazine to intact rats increased the total metabolism of testosterone and its conversion to both 5α-dihydro-

testosterone and 5α-androstanediol by DMBA-induced mammary tumours incubated in vitro. Perphenazine also stimulated tumour metabolism of testosterone and its conversion to 5α-reduced products in ovariectomised rats. However, the levels of total metabolism of testosterone and its conversion to 5α-dihydrotestosterone and 5α-androstanediol by the tumours of ovariectomised rats given perphenazine were similar to those of the intact control group and were not as high as those of the intact rats which received perphenazine. This set of circumstances appears to have been made possible by the lowered metabolism of testosterone found in ovariectomised-control compared to the intact-control group.

Treatment with CB 154 appeared to have no noticeable effects on the metabolism of testosterone by DMBA-induced tumours in that the values obtained for all parameters measured were similar to those of the intact control.

When the effects of the 2 treatment regimes which lower plasma prolactin were compared it was found that the metabolism of testosterone and its 5α-reduction was significantly higher in the tumours from intact rats given CB 154 compared to those from ovariectomised rats given corn oil.

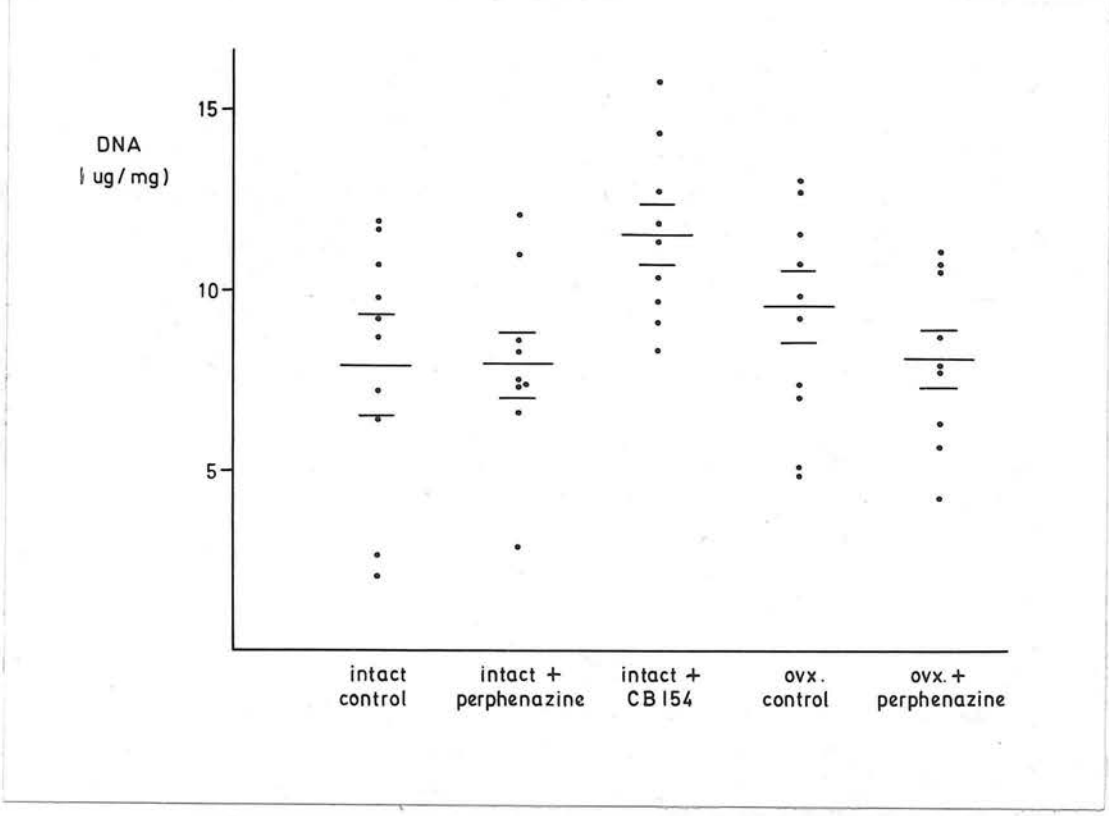
7. DNA content of tumours

The DNA content of tumours from the animal groups studied is shown in Fig. 29. The mean tumour DNA content of the control group does not differ significantly from the mean of other groups. The similarity between the DNA content of tumours from the groups receiving corn oil or perphenazine render it unlikely that the increased tumour metabolism of testosterone induced by perphenazine treatment is due to increased tumour cellularity.

8. Correlations between parameters determined on an individual basis

Since intergroup comparisons revealed that treatment regimes which elevated plasma prolactin and stimulated tumour growth also stimulated the capacity of tumours to metabolise testosterone to 5α-reduced products it was of

Figure 29. DNA levels in tumours used for steroid metabolism studies in the various treatment groups



See legend of Figure 25

interest to see if there were any correlations between these parameters within individual groups. It was also important to investigate possible relationships between testosterone metabolism and parameters such as the time from DMBA administration to the start of treatment and initial tumour size and growth since the latter were significantly greater in certain treatment groups than in the intact control group.

Interrelationships between the 16 parameters listed in Tables 18 - 22 have been examined by calculating the simple coefficient of correlation (r) for all paired combinations of interest (Table 16). When at least one of the pair of parameters was measured after treatment, the correlations were investigated on a within-group basis. When both of the parameters to be studied were measured prior to the start of treatment the correlations were investigated with data pooled from all five groups. The relationships between pretreatment parameters and tumour DNA content were also investigated in this manner as the levels of the latter appeared to be unaffected by the various treatment regimes.

The test for the simple coefficient of correlation, without excluding outlying values, was applied in preference to more sophisticated tests because it was desired to provide a general picture from a large volume of data. However, the number of observations for each parameter within a group was relatively small, and one or two extreme values in the presence of otherwise close groupings could greatly influence the determination of the coefficient of correlation. For this reason and the fact that the test was repetitively applied it is possible that some apparently significant correlations, especially at the 5% level, have been obtained purely by chance. Therefore, consideration should be given to trends throughout the groups or highly significant observations which may be supported by related findings rather than to odd individual values. Certain relevant observations deserve further comment.

The time between the administration of DMBA and the allocation to treatment groups varied considerably within each group, and was by chance

Legend for Table 16

The values presented in the table are the simple coefficients of correlation (r) for the corresponding parameters listed vertically and horizontally. Dependent on the appropriate degrees of freedom (d.f.), the further the value is displaced from zero towards +1 or -1 the greater the likelihood of a positive or negative correlation respectively. (* = $p < 0.05$, ** = $p < 0.01$)

Parameters

1 = days since DMBA

2 = days of treatment

3 = initial prolactin

4 = final prolactin

5 = oestradiol (at sacrifice)

6 = initial tumour size

7 = final tumour size

8 = initial tumour growth rate

9 = final tumour growth rate

10 = tumour DNA content

11 = testosterone metabolism

12 = 5α-DHT production

13 = 5α-Adiol production

14 = 5α-reduction

15 = $\frac{\text{5α-Adiol production}}{\text{5α-reduction}}$

16 = unaccounted-for metabolism

Table 16

Correlations between parameters measured in the five treatment groups

Parameter	9	10	11	12	13	14	15	16
1	.14	.12	.13	-.03	.47	.43	.30	-.49
4	-.30	-.08	.52	.51	.17	.29	-.23	.29
5	-.12	-.13	-.29	-.50	-.27	-.38	.32	.17
6	.14	.63*	-.17	-.08	-.02	.00	-.04	-.24
7	.68*	.76**	.09	-.05	.03	.02	-.12	.10
8	.22	.40	.27	.36	.05	.14	-.09	.17
9		.41	.27	-.22	-.03	-.08	.20	.52
10			.13	-.37	.19	.08	.49	.06
11				.27	.76**	.78**	.38	.21
12	Group 1. I C				.12	.36	-.69**	-.19
13	Comparison		d.f.			.97**	.58	-.43
14	10 vs 1 - 16		9				.37	-.45
15	all others		12					-.04
1	.02	-.03	.26	.42	.15	.36	-.45	-.42
4	-.22	.06	.01	.28	.13	.12	-.29	-.27
5	.42	.34	-.36	-.41	-.05	-.31	.37	.11
6	-.25	.40	.17	.33	-.06	.19	-.32	-.16
7	.44	-.01	.14	.14	-.16	.00	-.08	.25
8	-.17	.00	.53	.49	.20	.49	-.41	-.13
9		-.14	-.49	-.50	-.52	-.63*	.50	.67*
10			-.43	-.02	-.47	-.25	-.11	-.18
11				.75*	.78**	.93**	-.64*	-.50
12	Group 2. I + P				.32	.85**	-.98**	-.74*
13	Comparison		d.f.			.77**	-.18	-.51
14	10 vs 1 - 16		7				-.76**	-.78**
15	all others		8					.72*

Table 16 (continued)

Parameter	9	10	11	12	13	14	15	16
1	.01	-.05	-.02	-.12	.05	-.05	.09	.05
4	.06	-.37	.03	.46	.11	.41	-.39	-.53
5	.34	.42	-.12	-.25	-.40	-.49	.15	.57
6	.48	-.51	.42	.42	.33	.55	-.34	-.38
7	.79**	-.68*	.48	.48	.28	.55	-.40	-.33
8	.72*	-.58	.53	.48	.35	.61*	-.38	-.36
9		-.65	.30	.40	.16	.40	-.35	-.28
10			-.29	-.82**	-.30	-.77*	.76*	.80**
11				.06	.80**	.68*	.08	-.04
12	Group 3. I + B				-.10	.61*	-.98**	-.78**
13	Comparison		d.f.			.73**	.27	-.28
14	10 vs 1 - 16		7				-.46	-.76**
15	all others		9					.70*
1	-.29	.29	-.23	.13	.01	.04	-.18	-.55
2	.57	.33	.45	-.09	.18	.15	.30	.76**
4	-.33	.12	-.02	-.09	.19	.16	.01	-.28
6	-.77**	-.38	-.41	.14	-.40	-.34	-.64*	-.37
7	-.54	-.40	-.16	.30	-.23	-.15	-.53	-.11
8	-.51	-.22	-.19	.06	-.25	-.22	-.41	-.08
9		.21	.75*	.11	.56	.53	.64*	.83**
10			.14	-.38	.16	.08	.47	.18
11				.43	.92**	.91**	.55	.80**
12	Group 4. Ovx C				.46	.59	-.46	.05
13	Comparison		d.f.			.99**	.51	.52
14	all		8				.38	.49
15								.62

Table 16 (continued)

Parameter	9	10	11	12	13	14	15	16
1	.13	.36	.20	.29	.33	.34	-.08	-.41
4	.30	-.20	-.09	.34	.02	.14	-.45	-.47
6	-.28	.13	-.14	-.08	.07	.02	.06	-.28
7	.66*	-.31	-.21	.15	-.32	-.17	-.28	.02
8	-.28	-.03	-.18	-.21	-.16	-.20	-.01	.13
9		-.28	-.35	-.07	-.45	-.35	-.09	.17
10			-.11	.06	.16	.14	.13	-.50
11				.72*	.87**	.90**	-.44	-.25
12	Group 5. Ovx + P				.61	.82**	-.90**	-.57
13	Comparison		d.f.			.95**	-.28	-.60
14	10 vs 1 - 16		7				-.54	-.65*
15	all others		8					.42

Parameter	3	8	10	all data	
1	-.25	-.19	.07	Comparison	d.f.
3		.07	.17	1 vs 3	42
6		.81**	.14	10 vs 1 - 8	46
8			.11	all others	53

significantly higher in the intact perphenazine-treated group than in the intact control group. However, within each group this parameter showed no significant correlation with any of the other parameters. Initial tumour size and initial tumour growth, which were greater in rats being allocated to the intact-plus-CB 154 group and the ovariectomised control group, did not show significant correlations with any of the testosterone metabolism parameters in any of the five groups. From these results there is no evidence that the pretreatment differences between groups had any influence on subsequent measurements of testosterone metabolism. In the only group in which a noteworthy variation in the length of treatment period occurred, the ovariectomised control group, this parameter appeared to be unrelated to the metabolism of testosterone or to the formation of 5α-reduced products.

In each group the level of prolactin found in the plasma at sacrifice showed no significant correlation with final tumour growth rate, tumour DNA content or any of the testosterone metabolism parameters. However, the range of prolactin values within certain groups was very small due to the treatment regimes. The plasma oestradiol levels in each of the three intact groups also showed no significant correlation with any of the other parameters although it was observed that when plasma oestradiol levels were related to testosterone metabolism or the formation of 5α-reduced products, negative coefficients of correlation were always obtained. When final tumour size and final tumour growth rate or tumour DNA content were related to the parameters of testosterone metabolism no consistent correlations were observed throughout the groups.

As was suspected from the observation that in the two groups with higher initial tumour size initial tumour growth was also greater, there was a highly significant positive correlation between initial tumour growth and size, when all data were considered together.

When the relationships between testosterone metabolism parameters were investigated certain obvious correlations were observed. In each group the total level of testosterone metabolism showed a highly significant correlation with its major route of metabolism, 5α-reduction, and in turn this parameter showed a highly significant correlation with the production of its major component, 5α-androstanediol. However, only in the two perphenazine-treated groups did testosterone metabolism show a significant positive correlation with the net production of 5α-dihydrotestosterone and the relationship between the net production of 5α-dihydrotestosterone and 5α-androstanediol did not reach significance in any of the groups.

9. Effect of perphenazine on the metabolism of testosterone by tumours in vitro

The possibility of a direct stimulatory effect of perphenazine on the metabolism of testosterone was investigated by performing tumour incubations in the presence of perphenazine.

Two tumours taken from intact cycling rats in the dioestrus stage of the cycle were each divided into three portions which were then incubated in the presence of vehicle, 10^{-5}M perphenazine or 10^{-4}M perphenazine under the standard conditions described in the METHODS section. Perphenazine was first dissolved in a solution of ethanol/monopropylene glycol, so that addition of 50µl to the incubation mixture gave the desired final concentrations. After incubation, the levels of testosterone and its metabolites were assessed in the normal manner. The results are shown in Table 17.

The levels of 5α-dihydrotestosterone and 5α-androstanediol produced by the first tumour were relatively low and accounted for slightly less than half of the total testosterone metabolised. The amounts of testosterone metabolised, and the quantities of 5α-dihydrotestosterone and 5α-androstanediol recovered were remarkably similar in all three incubates so that perphenazine appeared to be without effect in this tumour. The metabolism of testosterone and the

Table 17

Effect of perphenazine on testosterone metabolism in vitro

	Addition to incubation medium	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n
1.	vehicle	52.25	2.78	15.74	18.52
	10^{-5}M perphenazine	50.33	3.17	15.97	19.14
	10^{-4}M perphenazine	56.50	3.85	14.28	18.13
2.	vehicle	33.12	11.18	37.11	48.29
	10^{-5}M perphenazine	39.28	10.32	28.08	38.40
	10^{-4}M perphenazine	45.43	7.50	25.45	32.95

The vehicle was ethanol/monopropylene glycol 1:1, of which a 50µl aliquot, with or without the requisite amount of perphenazine present, was added to a volume of 7.5ml prior to the start of the incubation.

formation of 5α-reduced products in the second tumour were higher and perphenazine appeared to have exerted a dose-related inhibitory effect on these parameters in this tumour.

10. Summary

The administration of perphenazine to intact rats bearing actively growing DMBA-induced tumours raised plasma prolactin levels, lowered plasma oestradiol levels, stimulated tumour growth rate and increased the capacity of tumours to metabolise testosterone by 5α-reduction in vitro. In intact rats given CB 154, plasma levels of prolactin, but not oestradiol, were lowered, tumour growth was inhibited, but there were no significant changes in the total metabolism of testosterone, or its conversion to 5α-dihydrotestosterone or 5α-androstanediol. Ovariectomy caused a fall in plasma levels of oestradiol and prolactin, immediate tumour regression and a slightly decreased capacity for testosterone metabolism by tumours. When ovariectomised rats received perphenazine, plasma oestradiol levels remained low, but prolactin levels were raised to the same levels as those of intact rats given perphenazine, tumour regression was delayed but not completely prevented and the capacity of tumours to metabolise testosterone by 5α-reduction was significantly higher than in tumours from the ovariectomised control group. Although the levels of testosterone metabolism and its conversion to 5α-reduced products were higher in tumours from the ovariectomised control animals these levels did not differ significantly from those of the intact control group, and were lower than those of intact rats given perphenazine.

When the correlations between parameters within each group were studied, there was no apparent relationship between plasma hormone levels, tumour growth rate and the metabolism of testosterone by tumours. A stimulatory effect of perphenazine has not been observed in vitro.

Legend for Tables 18 - 22

"Days since DMBA" refers to the length of time from the administration of carcinogen to the allocation to a treatment group.

"Initial" refers to values obtained immediately prior to the start of treatment, and "final" to those obtained at the end of treatment.

5α-red'n = 5α-DHT + 5α-Adiol.

unacc. metab. (unaccounted-for metabolism) = total metab. - 5α-red'n.

n.d. = not determined

Details of the treatment regimes are given in Table 10.

Table 18

Hormone levels and tumour parameters

of intact rats given corn oil

Rat	Days since DMBA	Days of treatment	Plasma prolactin (ng/ml) initial	Plasma prolactin (ng/ml) final	Plasma OE_2 (ng/100ml)	Position of tumour
311E	118	13	21	7	1.06	LAx
319E	113	12	11	52	0.42	RGr
332E	110	15	13	13	0.39	RAx
						RIn
381E	50	9	75	46	0.25	LAx
7H	60	11	78	45	0.76	RNk
						LAx
35H	84	15	7	15	0.34	RAx
301K	95	17	34	48	0.36	RGr
401K	43	12	12	22	1.92	RAx
323L	126	16	8	5	0.58	LAx
343L	100	14	21	5	0.81	LNk
						LGr
385L	82	12	52	23	0.58	RTh
mean	89.5	13.3	30.2	25.5	0.68	
s.d.	28.2	2.4	26.4	18.6	0.47	

Table 18 (continued)

Rat and tumour	Tumour size (cm^2) initial	Tumour size (cm^2) final	Tumour growth (cm^2/week) initial	Tumour growth (cm^2/week) final	DNA (μg/mg tissue)
311E LAx	2.60	3.00	0.57	0.16	2.65
319E RGr	3.30	3.99	0.69	0.37	n.d.
332E RAx	2.55	5.75	0.89	1.60	10.65
RIn	1.40	4.37	0.67	1.36	7.18
381E LAx	2.80	3.60	1.29	0.70	9.18
7H RNk	0.90	1.30	0.41	0.25	n.d.
LAx	1.50	1.40	0.81	-0.06	n.d.
35H RAx	2.55	3.06	0.35	0.37	2.07
301K RGr	4.50	6.51	2.08	0.87	8.66
401K RAx	1.82	3.40	0.81	0.92	6.45
323L LAx	4.10	4.83	1.16	0.42	11.70
343L LNk	2.76	3.94	0.84	0.63	9.76
LGr	5.25	6.96	1.06	0.93	16.81
385L RTh	2.92	4.56	0.80	0.96	11.85
mean	2.78	4.05	0.89	0.68	8.81
s.d.	1.22	1.66	0.43	0.46	4.21

Table 18 (continued)

Rat and tumour	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	5α-Adiol / 5α-red'n	% unacc. metab.
311E LAx	50.24	8.22	39.82	48.04	0.83	2.20
319E RGr	63.98	7.49	42.09	49.58	0.85	14.40
332E RAx	60.68	3.17	34.63	37.80	0.92	22.88
RIn	61.30	7.53	34.19	41.72	0.82	19.58
381E LAx	52.25	10.56	26.39	36.95	0.71	15.30
7H RNk	62.13	7.39	38.09	45.48	0.83	16.65
LAx	50.76	4.88	30.96	35.84	0.86	14.92
35H RAx	35.60	7.59	13.52	21.11	0.64	14.49
301K RGr	66.88	11.18	37.11	48.29	0.77	18.59
401K RAx	47.75	2.78	15.74	18.52	0.85	29.23
323L LAx	49.64	4.23	32.15	36.38	0.88	13.26
343L LNk	49.93	5.18	37.82	43.00	0.88	6.93
LGr	39.73	4.60	22.57	27.17	0.83	12.56
385L RTh	61.47	4.43	44.14	48.57	0.91	12.90
mean	53.74	6.37	32.09	38.46	0.83	15.28
s.d.	9.56	2.59	9.39	10.13	0.08	6.46

Table 19

Hormone levels and tumour parameters

of intact rats given perphenazine

Rat	Days since DMBA	Days of treatment	Plasma prolactin (ng/ml) initial	Plasma prolactin (ng/ml) final	Plasma OE_2 (ng/100ml)	Position of tumour
309E	116	14	23	87	0.10	RNk LIn
318E	111	14	63	90	0.61	RAx
333E	109	14	46	67	0.24	RNk
343E	158	17	44	19	0.92	RIn
357E	84	14	13	72	0.27	LAx
23H	87	16	56	81	0.12	RAx
60H	145	15	10	84	0.23	LGr
65H	116	16	5	100	0.56	LNk
87H	137	14	40	67	0.28	RGr
mean	118.1	14.9	33.3	74.1	0.37	
s.d.	24.9	1.2	21.2	23.4	0.27	

Table 19 (continued)

Rat and tumour	Tumour size (cm^2) initial	Tumour size (cm^2) final	Tumour growth (cm^2/week) initial	Tumour growth (cm^2/week) final	DNA (µg/mg tissue)
309E RNk	3.22	8.28	1.39	1.30	7.48
LIn	1.12	4.25	0.83	1.47	2.89
318E RAx	1.65	4.80	0.39	2.24	8.29
333E RNk	1.44	4.32	0.47	1.77	8.66
343E RIn	1.87	5.52	0.38	1.68	7.45
357E LAx	2.55	6.67	0.86	1.98	7.36
23H RAx	2.24	2.85	0.65	0.21	n.d.
60H LGr	1.92	4.00	0.46	0.88	6.61
65H LNk	2.34	4.94	0.97	1.12	12.10
87H RGr	2.21	4.40	0.61	1.23	11.00
mean	2.06	5.00	0.70	1.39	7.98
s.d.	0.60	1.52	0.32	0.58	2.63

Table 19 (continued)

Rat and tumour	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	$\frac{5\alpha\text{-Adiol}}{5\alpha\text{-red'n}}$	% unacc. metab.
309E RNk	82.22	32.75	49.18	81.93	0.60	0.29
LIn	87.04	23.92	57.44	81.36	0.70	5.68
318E RAx	48.02	4.27	37.39	41.66	0.90	6.36
333E RNk	53.01	5.53	34.27	39.80	0.86	13.21
343E RIn	71.26	10.30	53.80	64.10	0.84	7.16
357E LAx	58.28	4.09	33.10	37.19	0.89	21.09
23H RAx	70.83	13.02	57.08	70.28	0.81	0.55
60H LGr	68.93	28.23	40.30	68.53	0.59	0.40
65H LNk	66.21	20.29	43.79	64.08	0.68	2.13
87H RGr	70.69	30.25	40.23	70.48	0.57	0.21
mean	67.65	17.27	44.66	61.94	0.74	5.71
s.d.	12.07	11.19	9.16	16.62	0.13	6.58

Table 20

Hormone levels and tumour parameters

of intact rats given CB 154

Rat	Days since DMBA	Days of treatment	Plasma prolactin (ng/ml) initial	Plasma prolactin (ng/ml) final	Plasma OE_2 (ng/100ml)	Position of tumour
341L	95	13	71	4	1.60	LTh
345L	96	12	79	5	2.02	LAx LIn
351L	95	13	13	6	0.97	LTh
354L	130	12	14	3	1.08	LCh RIn
361L	97	14	38	6	1.24	LTh LAn
366L	72	12	5	9	0.60	RGr
384L	93	12	41	5	0.69	RAx RIn
mean	96.9	12.6	37.3	5.4	1.17	
s.d.	17.0	0.8	29.1	1.9	0.50	

Table 20 (continued)

Rat and tumour	Tumour size (cm^2) initial	Tumour size (cm^2) final	Tumour growth (cm^2/week) initial	Tumour growth (cm^2/week) final	DNA (µg/mg tissue)
341L LTh	5.00	3.00	1.46	-1.08	15.84
345L LAx	2.55	1.65	0.51	-1.97	14.41
LIn	10.00	11.70	2.77	+0.99	9.71
351L LTh	7.80	6.67	2.42	-0.61	12.73
354L LCh	5.25	7.13	2.50	+1.10	9.08
RIn	3.52	2.04	0.62	-0.86	n.d.
361L LTh	5.72	4.00	1.12	-0.86	11.42
LAn	4.56	2.88	0.70	-0.84	10.37
366L RGr	8.20	9.24	3.14	+0.58	8.36
384L RAx	4.20	6.35	1.81	+1.60	11.87
RIn	3.96	2.56	1.61	-0.84	n.d.
mean	5.52	5.20	1.70	-0.25	11.54
s.d.	2.25	3.27	0.92	1.13	2.49

Table 20 (continued)

Rat and tumour	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	5α-Adiol / 5α-red'n	% unacc. metab.
341L LTh	50.08	3.98	27.36	31.34	0.87	18.74
345L LAx	55.55	3.71	33.04	36.75	0.90	18.70
LIn	61.97	8.94	39.51	48.45	0.82	13.52
351L LTh	63.15	5.27	49.00	54.27	0.90	8.88
354L LCh	56.76	13.29	35.61	48.90	0.73	7.87
RIn	56.27	5.60	41.09	46.69	0.88	9.58
361L LTh	47.91	4.92	35.00	39.92	0.88	7.99
LAn	52.35	14.11	32.36	46.47	0.70	5.88
366L RGr	56.82	17.90	37.41	55.31	0.67	1.51
384L RAx	55.53	4.00	37.05	41.05	0.90	14.48
RIn	60.47	4.59	42.79	47.38	0.90	13.09
mean	56.07	7.85	37.29	45.14	0.83	10.93
s.d.	4.50	4.99	5.80	7.25	0.09	5.32

Table 21

Hormone levels and tumour parameters

of rats ovariectomised and given corn oil

Rat	Days since DMBA	Days of treatment	Plasma prolactin (ng/ml) initial	Plasma prolactin (ng/ml) final	Plasma OE_2 (ng/100ml)	Position of tumour
306K	89	14	28	2	0.33	LNk
313K	90	11	34	4	0.26	LAn
330K	106	14	52	12	0.10	RTh
407K	142	7	10	7	0.15	LAx
26L	113	8	8	4	0.07	RNk
29L	113	8	4	4	0.05	LNk
31L	108	6	11	8	0.12	LAx
48L	110	8	116	11	0.08	LIn
65L	98	9	30	7	0.04	RTh
85L	104	11	78	8	0.00	LSh
mean	107.3	9.6	37.1	6.7	0.12	
s.d.	14.9	2.8	35.9	3.2	0.10	

Table 21 (continued)

Rat and tumour	Tumour size (cm^2) initial	Tumour size (cm^2) final	Tumour growth (cm^2/week) initial	Tumour growth (cm^2/week) final	DNA (µg/mg tissue)
306K LNk	4.83	3.30	1.39	-0.77	11.75
313K LAn	7.75	6.16	1.81	-1.01	5.08
330K RTh	4.80	2.38	1.69	-1.21	14.00
407K LAx	6.75	3.80	1.48	-2.95	12.54
26L RNk	4.84	2.56	0.86	-2.00	4.86
29L LNk	6.38	3.57	0.76	-2.46	9.83
31L LAx	5.20	3.08	0.73	-2.47	13.73
48L LIn	6.90	2.85	0.93	-3.54	7.36
65L RTh	16.34	9.86	4.02	-5.04	7.01
85L LSh	7.36	3.20	2.24	-2.65	9.22
mean	7.12	4.08	1.59	-2.41	9.54
s.d.	3.43	2.29	0.99	1.28	3.41

Table 21 (continued)

Rat and tumour	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	5α-Adiol / 5α-red'n	% unacc. metab.
306K LNk	41.40	1.57	13.57	15.14	0.90	26.26
313K LAn	68.33	9.05	36.62	45.67	0.80	22.66
330K RTh	69.07	4.72	42.26	46.98	0.90	22.09
407K LAx	36.68	6.05	22.90	28.95	0.79	7.73
26L RNk	43.93	3.98	28.47	32.45	0.88	11.48
29L LNk	30.11	5.84	17.83	23.72	0.75	6.39
31L LAx	42.83	2.57	28.91	31.48	0.92	11.35
48L LIn	23.00	4.01	13.68	17.69	0.77	5.31
65L RTh	23.07	4.17	12.31	16.48	0.75	6.59
85L LSh	22.71	3.29	13.00	16.29	0.80	6.42
mean	40.11	4.53	22.96	27.49	0.83	12.63
s.d.	17.18	2.09	10.74	11.84	0.07	7.96

Table 22

Hormone levels and tumour parameters

of rats ovariectomised and given perphenazine

Rat	Days since DMBA	Days of treatment	Plasma prolactin (ng/ml) initial	Plasma prolactin (ng/ml) final	Plasma OE_2 (ng/100ml)	Position of tumour
378E	69	14	46	92	0.22	RCh RAx
380E	122	14	35	81	0.12	LGr
397E	100	14	12	105	0.29	RCh RAx
26H	98	14	17	44	0.06	RAx
45H	71	14	88	102	0.09	RAx
269K	97	14	55	128	0.15	LAx
297K	96	14	8	63	0.53	RAx LAx
mean	93.3	14	37.3	87.9	0.21	
s.d.	18.3	0	28.5	28.0	0.16	

Table 22 (continued)

Rat and tumour	Tumour size (cm^2) initial	Tumour size (cm^2) final	Tumour growth (cm^2/week) initial	Tumour growth (cm^2/week) final	DNA (µg/mg tissue)
378E RCh	2.80	2.33	1.61	-1.04	7.91
RAx	3.30	4.43	1.72	+0.50	4.21
380E LGr	3.00	2.66	0.58	-0.16	11.05
397E RCh	2.95	4.75	0.55	0.00	8.70
RAx	1.57	1.72	1.16	-0.45	n.d.
26H RAx	3.91	3.48	1.09	-0.55	10.55
45H RAx	3.20	3.24	1.68	-0.50	10.77
269K LAx	3.04	4.51	0.52	+0.83	6.32
297K RAx	3.15	2.60	0.55	-1.07	5.64
LAx	1.40	3.45	0.61	+0.84	7.79
mean	2.83	3.32	1.01	-0.16	8.10
s.d.	0.77	1.01	0.51	0.70	2.42

Table 22 (continued)

Rat and tumour	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	5α-Adiol / 5α-red'n	% unacc. metab.
378E RCh	77.73	8.83	54.74	63.57	0.86	14.16
RAx	54.88	8.49	23.64	32.13	0.74	22.75
380E LGr	62.32	10.94	48.74	59.68	0.82	2.64
397E RCh	78.35	26.43	45.11	71.54	0.63	6.81
RAx	65.10	15.13	38.55	53.68	0.72	11.42
26H RAx	54.00	5.20	33.76	38.96	0.87	15.04
45H RAx	28.34	2.08	10.84	12.92	0.84	15.42
269K LAx	42.19	5.08	27.19	32.07	0.85	10.12
297K RAx	62.49	5.54	32.76	38.30	0.86	24.19
LAx	51.06	1.99	16.90	18.89	0.89	32.17
mean	57.64	8.97	33.22	42.17	0.81	15.47
s.d.	15.26	7.33	14.02	19.39	0.08	8.78

IV. Testosterone metabolism by DMBA-induced mammary tumours at various stages of the oestrous cycle

From the results described in the previous section it appeared that a change in hormonal status of approximately two weeks duration could result in an alteration of the capacity of DMBA-induced mammary tumours to metabolise testosterone in comparison with that of tumours taken from control rats in the dioestrus stage of the oestrous cycle. Since the secretion of pituitary and ovarian hormones undergoes cyclic changes it was decided to measure testosterone metabolism by tumours removed from untreated rats on different days of the oestrous cycle.

1. Experimental design

For this study actively growing tumours were taken from rats which had just shown at least three regular 4-day cycles. Immediately prior to tumour excision, at between 1030 and 1200 hours, blood was removed from the tail vein for the assay of plasma prolactin and from the aorta for the assay of plasma oestradiol. Tumours were divided into portions for histological examination, measurement of DNA content and testosterone metabolism under the standard conditions described in the METHODS section. Animals were allocated for sacrifice on the different days of the cycle on the basis of tumour size, tumour growth and "days since DMBA" so that, as far as possible, the mean values of these parameters were similar in all four groups.

The individual results from each group are given in Tables 24 - 27. An exceptionally high level of plasma oestradiol was found in one rat of the metoestrus group and two tumours, one from the oestrus group and one from the dioestrus group, showed extremely low formation of 5α-reduced products. These outlying values, along with that of testosterone metabolism in the first-mentioned tumour which was also exceptionally low, were not used for the

intergroup comparisons by variance analysis (Table 23), or for the determination of the simple coefficient of correlation between parameters (Table 28).

2. Plasma levels of oestradiol and prolactin

At the particular time of sampling employed no significant differences in the mean plasma prolactin levels on the various days of the oestrous cycle were observed. In contrast the levels of oestradiol were significantly higher at proestrus than on all other days of the cycle and the oestradiol levels at dioestrus were significantly higher than at oestrus and metoestrus.

3. Metabolism of testosterone by tumours

Total testosterone metabolism, 5α-dihydrotestosterone production, 5α-androstanediol production and 5α-reduction in tumours taken at different stages of the cycle were compared by variance analysis (Table 23). There were no significant differences between the different groups with regard to any of the parameters. It can be seen from Figures 30 - 33 which show the outlying values omitted from the variance analysis that the inclusion of these figures would not have altered the outcome of the statistical analysis.

Although a statistically significant difference between groups could not be detected it was noticeable that tumours removed at metoestrus tended to show high testosterone metabolism (>50%) and high 5α-reduction (>45%). In contrast five of the six tumours removed at proestrus had 5α-reductase values of below 45%.

4. Correlations between parameters determined on an individual basis

The numbers in each group were too small to justify separate group analysis so all data were pooled to determine the simple coefficients of correlation (r) between parameters as described in the previous section. Since the test was again repetitively applied the reservations stated previously also apply.

Table 23

Hormone levels and tumour parameters during the oestrous cycle - summary of results

Stage of cycle	Days since DMBA	Plasma prolactin (ng/ml)	Plasma oestradiol (ng/100ml)	Tumour growth (cm^2/week)	Tumour size (cm^2)
Proestrus	139	37	4.41**	1.46	5.33
Oestrus	126	40	0.24	1.12	5.06
Metoestrus	126	45	0.29	1.20	5.12
Dioestrus	120	32	0.91*	0.88	4.62
within group s.d.	25.8	18.6	0.42	0.62	2.58
within group d.f.	19	19	18	23	23

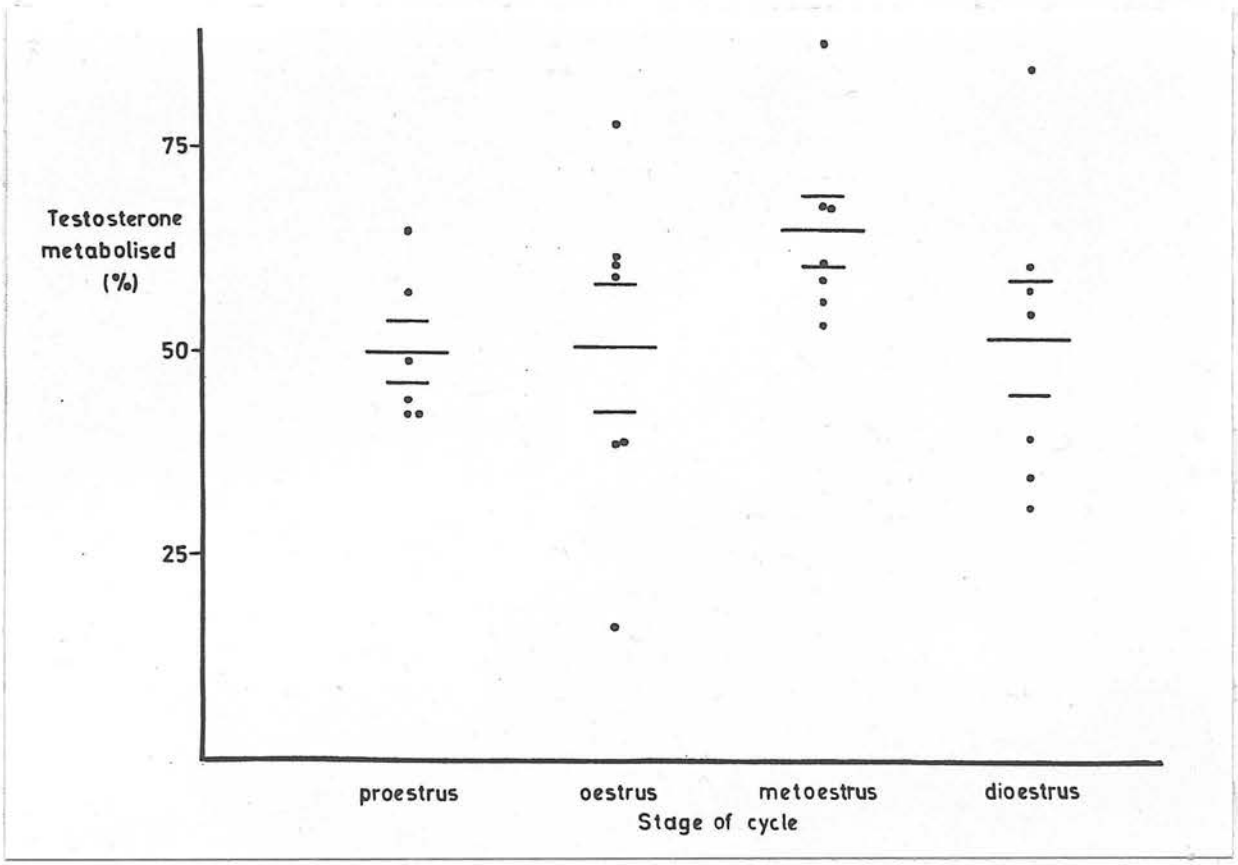
Stage of cycle	DNA (µg/mg tissue)	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	5α-Adiol / 5α-red'n
Proestrus	9.10	50.02	8.76	29.25	38.01	0.77
Oestrus	10.48	56.39	7.24	40.96	48.20	0.84
Metoestrus	11.47	65.16	12.90	44.38	57.27	0.78
Dioestrus	8.91	52.20	7.35	39.93	47.27	0.85
within group s.d.	2.72	14.26	5.27	11.95	15.25	0.08
within group d.f.	22	22	21	21	21	23

Intercomparisons of the mean values of all parameters were made using variance analysis plus the Student-Newman-Keuls test.

* and ** = significantly different from values at other stages of the cycle ($p < 0.05$ and $p < 0.01$ respectively).

Figure 30. Testosterone metabolism by DMBA-induced mammary tumours removed at different stages of the oestrous cycle

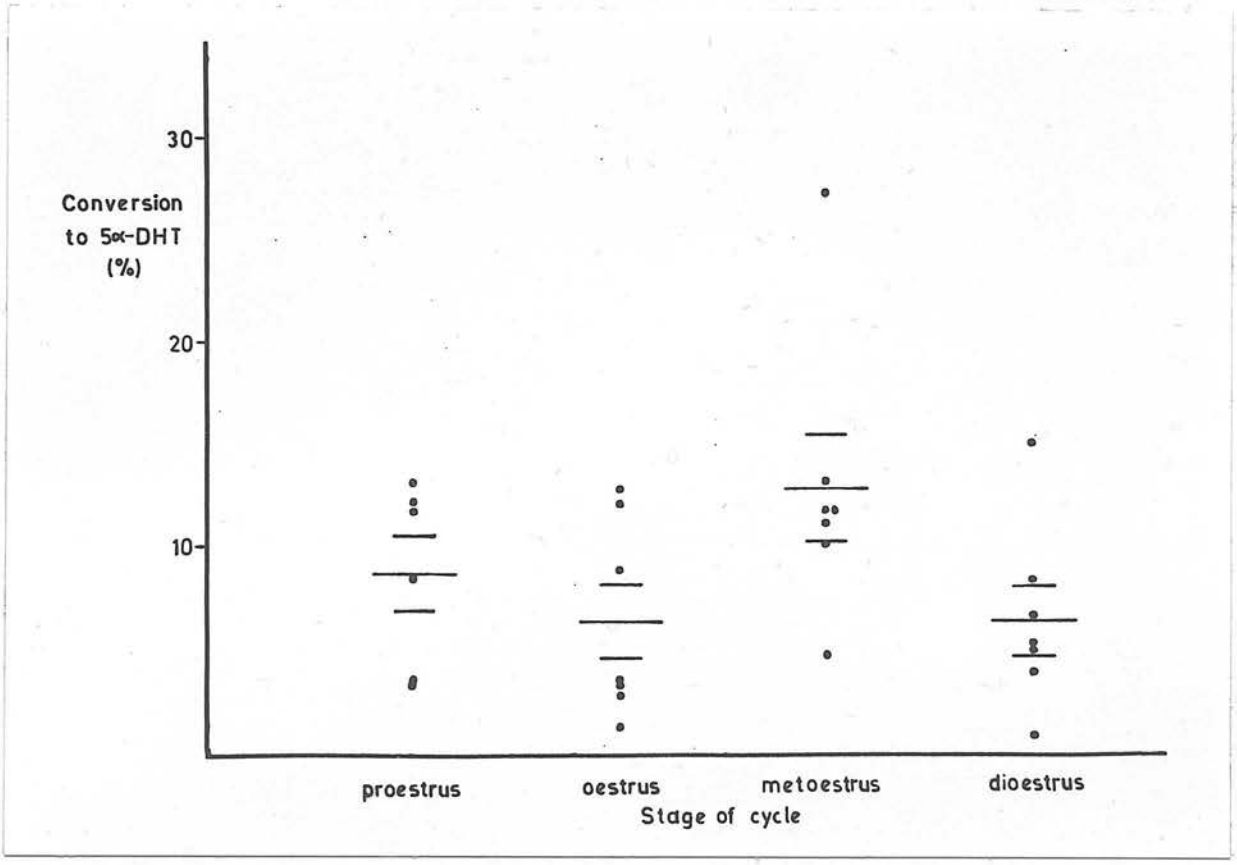
(a) Total metabolism of testosterone



Tumours were removed between 1030 and 1200 hours on the different days of the oestrous cycle and incubated as described in the METHODS section. Each point represents the value from an individual tumour. Horizontal bars indicate the means and s.e. means for each group. Statistical analyses are given in Table 23.

Figure 31. Testosterone metabolism by DMBA-induced mammary tumours removed at different stages of the oestrous cycle

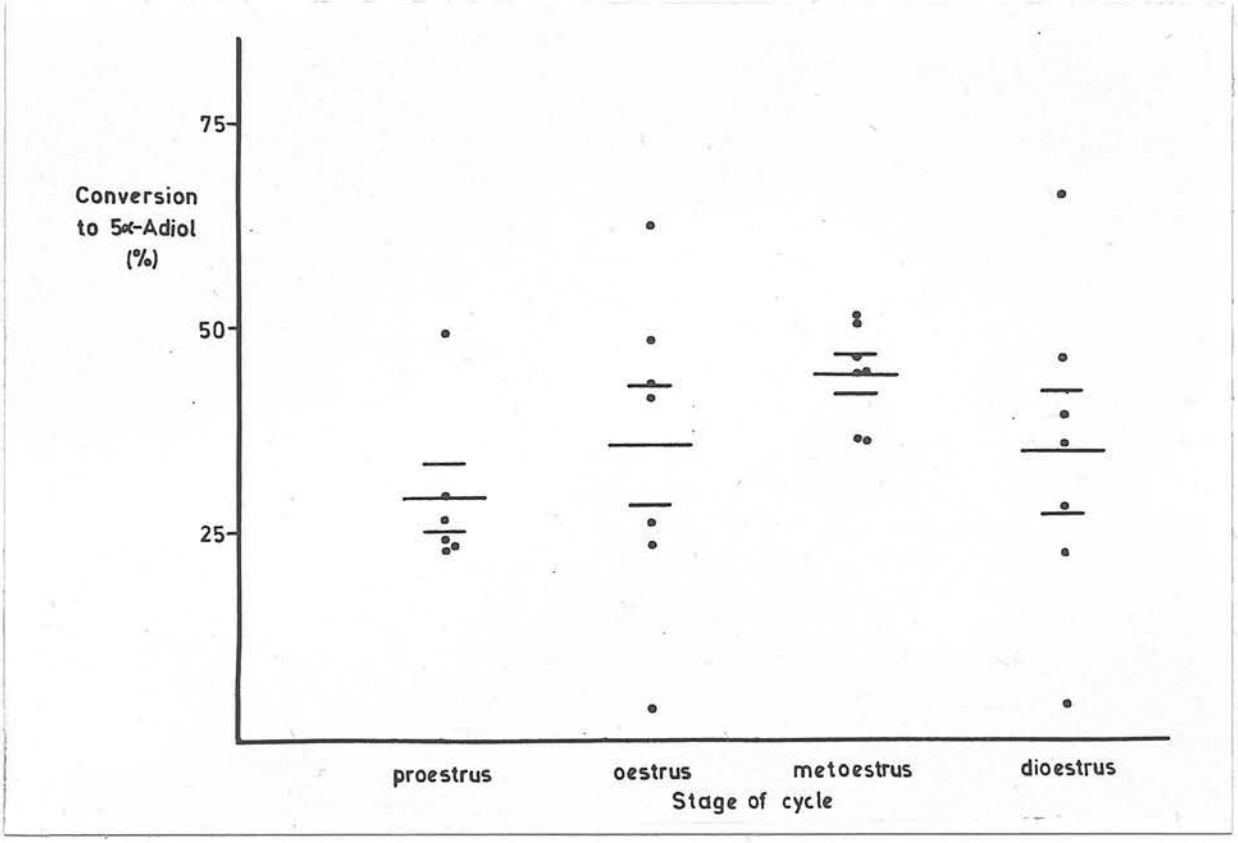
(b) Conversion to 5α-dihydrotestosterone



See legend of Figure 30

Figure 32. Testosterone metabolism by DMBA-induced mammary tumours removed at different stages of the oestrous cycle

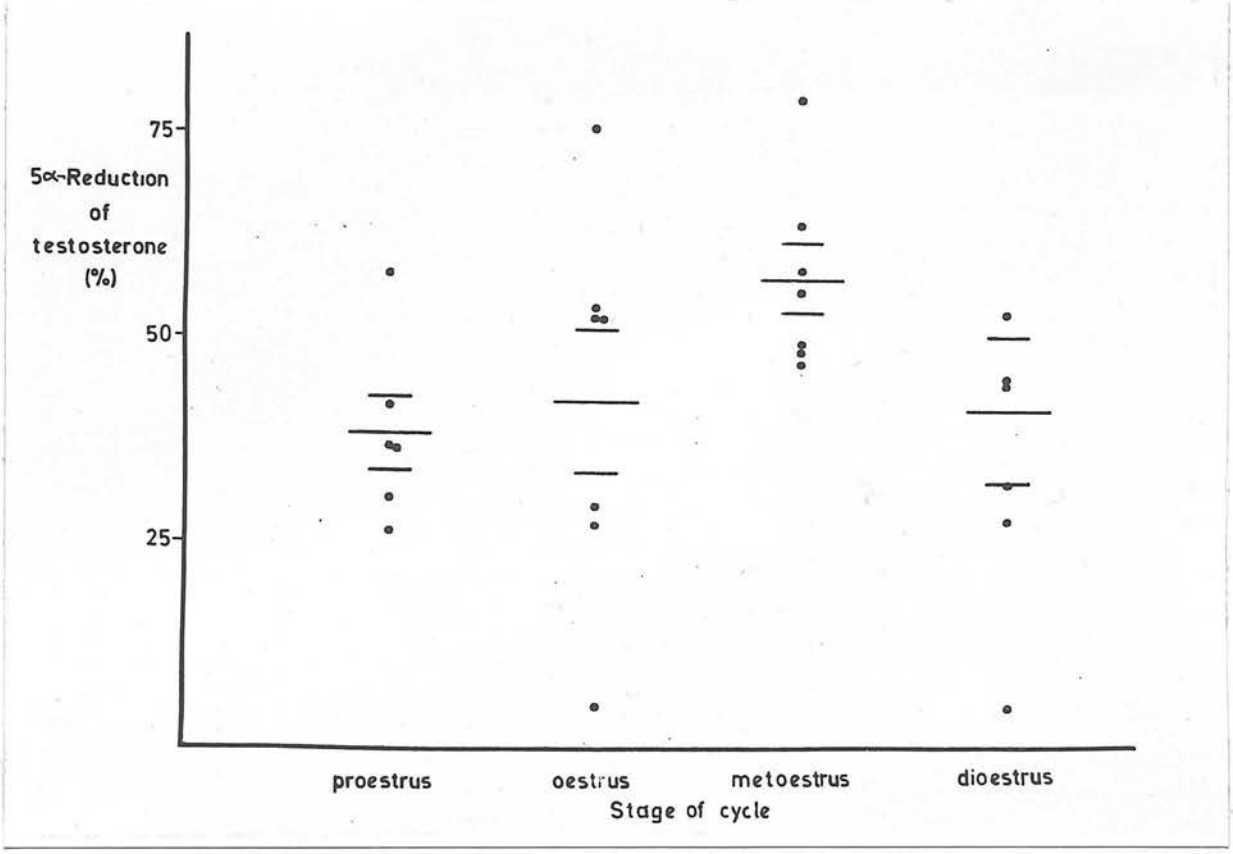
(c) Conversion to 5α-androstanediol



See legend of Figure 30

Figure 33. Testosterone metabolism by DMBA-induced mammary tumours removed at different stages of the oestrous cycle

(d) 5α-reduction of testosterone



See legend of Figure 30

In this group of tumours there also appeared to be no significant correlation between the parameters of testosterone metabolism and "days since DMBA", tumour size, tumour growth and tumour DNA content (Table 28).

Plasma levels of prolactin and oestradiol showed a significant negative correlation with 5α-androstanediol production and the closely related 5α-reduction, although the relationship with prolactin just achieved significance at the 5% level.

As noted in the previous section there was a strong positive correlation between total testosterone metabolism, 5α-reduction and 5α-dihydrotestosterone production. In this series of tumours 5α-dihydrotestosterone production showed a positive correlation with testosterone metabolism, 5α-androstanediol production and therefore 5α-reduction.

5. Summary

When inter-group comparisons of the various parameters were made, only the expected differences in plasma levels of oestradiol were significant. Although the capacity of tumours to metabolise testosterone by 5α-reduction was not significantly different between the four stages of the cycle, the numerical differences between the metoestrus and proestrus stages may have attained significance with a greater number of observations.

Table 24

Hormone levels and tumour parameters during the oestrous cycle - proestrus stage

Rat	Days since DMBA	Plasma prolactin (ng/ml)	Plasma OE_2(ng/100ml)	Position of tumour	Tumour growth (cm^2/week)	Tumour size (cm^2)
6M	137	49	4.25	RGr	0.75	4.16
9M	116	38	4.86	LCh	2.44	11.20
13M	181	43	3.39	RGr	3.00	6.00
33M	172	37	5.36	RIn	0.68	3.60
				LNk	0.52	2.04
153M	88	17	4.19	RIn	1.38	4.96
mean	139	37	4.41		1.46	5.33
s.d.	38.7	12.1	0.74		1.03	3.17

(Continued) Rat and tumour	DNA (µg/mg tissue)	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	$\frac{5\alpha\text{-Adiol}}{5\alpha\text{-red'n}}$
6M RGr	2.41	57.28	13.17	23.40	36.57	0.64
9M LCh	7.33	42.36	11.83	29.57	41.40	0.71
13M RGr	6.91	42.27	12.24	23.78	36.02	0.66
33M RIn	11.72	44.33	3.28	22.88	26.16	0.87
LNk	12.64	48.99	3.48	26.65	30.13	0.88
153M RIn	13.63	64.86	8.58	49.22	57.80	0.85
mean	9.10	50.02	8.76	29.25	38.01	0.77
s.d.	4.30	9.22	4.45	10.10	11.06	0.11

Table 25

Hormone levels and tumour parameters during the oestrous cycle - oestrus stage

Rat	Days since DMBA	Plasma prolactin (ng/ml)	Plasma OE_2(ng/100ml)	Position of tumour	Tumour growth (cm^2/week)	Tumour size (cm^2)
390L	160	52	0.51	LAx	1.03	6.72
10M	130	25	0.27	RAx	1.13	2.52
19M	134	48	0.00	RAx	1.45	6.51
23M	107	70	0.34	LAx	1.28	5.25
115M	112	14	0.11	RTh	1.21	3.42
				RAn	1.19	6.50
132M	114	33	0.18	LIn	0.57	4.50
mean	126	40	0.24		1.12	5.06
s.d.	20	20	0.18		0.28	1.65

(Continued) Rat and tumour	DNA (µg/mg tissue)	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	$\frac{5\alpha\text{-Adiol}}{5\alpha\text{-red'n}}$
390L LAx	7.29	16.26*	1.27*	3.50*	4.77*	0.73
10M RAx	8.47	60.66	8.97	43.13	52.10	0.83
19M RAx	10.70	38.90	3.31	23.51	26.82	0.88
23M LAx	10.44	39.13	2.80	26.25	29.05	0.90
115M RTh	12.73	78.38	12.80	62.77	75.57	0.83
RAn	12.55	59.54	12.09	41.53	53.62	0.77
132M LIn	11.20	61.71	3.45	48.59	52.04	0.93
mean	10.48	50.65	6.39	35.61	42.00	0.84
s.d.	2.01	20.51	4.79	19.42	23.27	0.07

Table 26

Hormone levels and tumour parameters during the oestrous cycle - metoestrus stage

Rat	Days since DMBA	Plasma prolactin (ng/ml)	Plasma OE_2 (ng/100ml)	Position of tumour	Tumour growth (cm^2/week)	Tumour size (cm^2)
391L	146	22	0.28	RIn	0.68	4.14
27M	116	38	0.25	RAn	2.19	6.44
				LGr	0.63	4.37
31M	120	45	0.18	LAx	1.70	3.84
139M	135	44	4.35*	LTh	1.28	6.12
151M	144	38	0.43	LNk	1.10	5.70
164M	95	85	0.33	RCh	0.80	5.25
mean	126	45	0.97		1.20	5.12
s.d.	20	21	1.66		0.58	1.02

(Continued) Rat and tumour	DNA (µg/mg tissue)	total metab.	Testosterone metabolism (%) 5α-DHT	5α-Adiol	5α-red'n	$\frac{5\alpha\text{-Adiol}}{5\alpha\text{-red'n}}$
391L RIn	8.05	68.55	11.80	46.55	58.35	0.80
27M RAn	12.39	68.11	13.18	50.65	63.83	0.79
LGr	10.90	88.49	27.53	51.71	79.24	0.65
31M LAx	9.02	61.28	11.14	44.39	55.53	0.80
139M LTh	11.40	53.71	10.13	36.60	46.73	0.73
151M LNk	15.16	56.69	11.73	36.38	48.11	0.76
164M RCh	13.34	59.31	4.76	44.36	49.12	0.90
mean	11.47	65.16	12.90	44.38	57.27	0.78
s.d.	2.45	11.67	7.00	6.09	11.48	0.07

Table 27

Hormone levels and tumour parameters during the oestrous cycle - dioestrus stage

Rat	Days since DMBA	Plasma prolactin (ng/ml)	Plasma OE_2(ng/100ml)	Position of tumour	Tumour growth (cm^2/week)	Tumour size (cm^2)
317E	96	67	1.26	RNk	1.16	3.15
5H	107	36	0.67	LCh	0.56	4.20
14M	118	21	0.96	LAx	1.43	12.80
45M	100	28	0.52	RIn	0.84	2.40
113M	155	18	0.54	LGr	0.77	3.96
127M	141	21	1.50	RNk	1.16	3.15
				RAx	0.21	2.70
mean	120	32	0.91		0.88	4.62
s.d.	24	18	0.40		0.41	3.66

(Continued) Rat and tumour	DNA (µg/mg tissue)	Testosterone metabolism (%) total metab.	5α-DHT	5α-Adiol	5α-red'n	$\frac{5\alpha\text{-Adiol}}{5\alpha\text{-red'n}}$
317E RNk	n.d.	39.97	3.90	28.27	32.17	0.88
5H LCh	7.75	31.26	0.81*	4.06*	4.87*	0.83
14M LAx	7.03	58.00	8.36	35.93	44.29	0.81
45M RIn	8.49	60.67	6.58	46.36	52.94	0.88
113M LGr	10.61	85.33	15.04	66.66	81.70	0.82
127M RNk	9.40	35.20	4.97	22.65	27.62	0.82
RAx	10.16	54.96	5.23	39.69	44.92	0.88
mean	8.91	52.20	6.41	34.80	41.22	0.85
s.d.	1.40	18.65	4.46	19.62	23.76	0.03

Legend for Tables 24 - 27

"Days since DMBA" refers to the length of time from the administration of the carcinogen to the allocation to a treatment group.

5α-red'n = 5α-DHT + 5α-Adiol

n.d. = not determined

* = values not listed for variance analysis or the determination of the simple coefficient of correlation.

Parameters listed in Table 28

1 = days since DMBA

2 = plasma prolactin

3 = plasma oestradiol

4 = tumour growth

5 = tumour size

6 = tumour DNA content

7 = testosterone metabolism

8 = 5α-DHT production

9 = 5α-Adiol production

10 = 5α-reduction

11 = $\frac{\text{5α-Adiol production}}{\text{5α-reduction}}$

Table 28

Correlations between parameters measured in untreated rats throughout the oestrous cycle

Parameter	2	3	4	5	6	7	8	9	10	11
1	-.19	.40*	.01	-.16	-.14	-.19	.00	-.35	-.27	-.25
2		-.01	.10	.01	-.04	-.39	-.26	-.41*	-.41*	.09
3			.11	.03	-.22	-.37	-.16	-.50*	-.45*	-.18
4				.54	-.19	-.29	-.15	-.20	-.11	-.41*
5					-.15	-.14	.14	-.14	-.07	-.31
6						.20	-.10	.35	.24	.41*
7							.70**	.90**	.96**	-.19
8								.47*	.72**	-.78**
9									.95**	.09
10										-.20

Data from the four stages of the oestrous cycle were pooled to calculate the simple coefficient of correlation (r) for the corresponding parameters listed vertically and horizontally.

* = $p < 0.05$, ** = $p < 0.01$, d.f. = 23

V. Conversion of testosterone to androst-4-ene-3α, 17β-diol by DMBA-induced rat mammary tumours

In addition to testosterone, 5α-dihydrotestosterone and 5α-androstanediol (3α), authentic androst-4-ene-3α, 17β-diol was also added as a non-radioactively labelled carrier (500μg) to the last 12 incubates worked up for the measurement of testosterone metabolism by rat mammary tumours removed at different stages of the oestrous cycle (Section IV).

The initial separation of steroids extracted from these incubates was performed in solvent system Ib in preference to Ia to achieve a greater separation of androst-4-ene-3α, 17β-diol from the 5α-androstanediol band. The band containing androst-4-ene-3α, 17β-diol, visualised under u.v. light, was scraped from the plate and the eluate incubated with manganese dioxide, a material reported to preferentially oxidise allylic alcohols (see METHODS). The products of this reaction were run in solvent system III and the two bands corresponding to testosterone and 4-androstenedione (identified under u.v. light by characteristic colour and mobilities identical to those of the authentic steroids) were removed for measurement of specific activity. The testosterone derivative accounted for approximately half of the carrier added and 4-androstenedione derivative for about an eighth. When these plates were scanned it was observed that in addition to radioactive peaks running with testosterone and 4-androstenedione a peak ran with the same mobility as authentic androst-4-ene-3α, 17β-diol, indicating that the reaction with manganese dioxide was incomplete.

The specific activity of the testosterone derivative, designated "testosterone 1" in Table 29, was consistently higher than that of the 4-androstenedione derivative. Therefore, the acetate was prepared from testosterone and run in the standard manner. The specific activity of testosterone acetate was lower than that of testosterone 1 and similar to that of

Table 29

Evidence for the identification of androst-4-ene-3α, 17β-diol

Rat and tumour	Specific activities of derivatives					
	a Δ^4Dione	b Testo 1	c Testo Ac	d Testo 2	Average of a, c and d	% of total d.p.m.
33M LNk	1,997	2,171	2,002	2,084	2,028	7.25
33M RIn	1,853	2,087	1,970	2,025	1,949	6.76
113M LGr	351	694	325	324	333	1.09
115M RTh	449	464	422	437	436	1.45
115M RAn	487	507	465	472	475	1.61
127M RNk	975	1,045	1,033	1,080	1,029	3.24
127M RAx	1,241	1,345	1,289	1,273	1,268	4.05
132M LIn	1,315	1,340	1,296	1,302	1,304	4.26
139M LTh	616	688	626	611	618	1.93
151M LNk	680	734	651	666	666	2.19
153M RIn	665	704	617	678	653	2.18
164M RCh	874	1,024	902	917	898	3.10

After the initial separation of precursor and metabolites in solvent system Ib, androst-4-ene-3α, 17β-diol was oxidised with manganese dioxide to yield 4-androstenedione and testosterone 1 which were separated in solvent system III. Testosterone 1 was converted to testosterone acetate which was then hydrolysed to yield testosterone 2. Testosterone 1 was judged impure and not used for calculation of the percentage conversion.

the 4-androstenedione derivative. Hydrolysis of testosterone acetate back to testosterone (testosterone 2) did not alter the specific activity. Since the specific activities of the 4-androstenedione, testosterone acetate and testosterone derivatives appeared to be constant these were averaged to calculate the percentage formation of androst-4-ene-3α, 17β-diol. It was assumed from the higher specific activity of testosterone 1 that impurities were present, possibly products of the oxidation of contaminant 5α-androstanediol by manganese dioxide.

It was of interest to compare the conversion of testosterone to androst-4-ene-3α, 17β-diol with the formation of other products and with the total metabolism of testosterone. In Table 30 tumours are listed in descending order of their ability to convert testosterone to androst-4-ene-3α, 17β-diol. It can be seen that pairs of tumours from individual rats converted testosterone to androst-4-ene-3α, 17β-diol to a similar extent. The levels of 5α-dihydrotestosterone but not those of 5α-androstanediol recovered were also similar in the same pairs of incubates.

The quantities of androst-4-ene-3α, 17β-diol produced by these tumours were remarkably high, in some cases higher than the net production of 5α-dihydrotestosterone. The higher production of androst-4-ene-3α, 17β-diol appears to have occurred in tumours in which larger quantities of total testosterone metabolism were unaccounted for by 5α-reduction.

Before its identification there was an impression gained that the radioactive peak now attributed to androst-4-ene-3α, 17β-diol was greater when those of the 5α-reduced metabolites were smaller than usual. The results from these 12 tumours would seem to support this notion, with the greatest 5α-reduction occurring in the tumours with the lowest formation of androst-4-ene-3α, 17β-diol.

By the simple coefficient of correlation test the formation of androst-4-ene-3α, 17β-diol showed a significant positive correlation with testosterone

Table 30

Metabolism of testosterone to androst-4-ene-3α, 17β-diol by DMBA-induced rat mammary tumours

Rat and tumour	total metab.	Testosterone metabolism % 5α-DHT	5α-Adiol	5α-red'n	Total - 5α-red'n	Δ^4diol(3α)
33M LNk	46.99	3.48	26.65	30.13	16.86	7.25
33M RIn	44.33	3.28	22.88	26.16	18.17	6.76
132M LIn	61.71	3.45	48.59	52.04	9.67	4.26
127M RAx	54.96	5.23	39.69	44.92	10.04	4.05
127M RNk	35.20	4.97	22.65	27.62	7.58	3.24
164M RCh	59.31	4.76	44.36	49.12	10.19	3.10
151M LNk	56.69	11.73	36.38	48.11	8.58	2.19
153M RIn	64.86	8.58	49.22	57.80	7.06	2.18
139M LTh	53.71	10.13	36.60	46.73	6.98	1.93
115M RAn	59.54	12.09	41.53	53.62	5.92	1.61
115M RTh	78.38	12.80	62.77	75.57	2.81	1.45
113M LGr	85.33	15.04	66.66	81.70	3.63	1.09

All of these tumours comprised part of the study in Section IV and are listed above in descending order of their ability to form androst-4-ene-3α, 17β-diol (Δ^4diol (3α)). Total - 5α-red'n = the metabolism of testosterone not accounted for by the formation of 5α-DHT and 5α-Adiol.

metabolism unaccounted-for by 5α-reduction and a significant negative correlation with 5α-reduction itself ($p < 0.05$).

VI. The effect of 5α-reduced metabolites on the growth of DMBA-induced rat mammary tumours

Since 5α-reduced metabolites appear to be responsible for mediating the effects of testosterone in many androgen responsive tissues, information on the effect of these steroids on mammary tumour growth might help in the understanding of the significance of the metabolism of testosterone by tumours. Although the effects of testosterone propionate and 5α-androstane - 3α,17β-diol dipropionate on the growth of DMBA-induced mammary tumours have been studied, the effects of synthetic C-19 steroids have received more attention (Griswold, Skipper, Laster, Wilcox & Schabel, 1966; Heise & Gorlich, 1966; Teller, Stock, Stohr, Merker, Kaufman, Escher & Bowie, 1966). No study has reported the effect of different 5α-reduced steroids on DMBA-induced rat mammary tumours. It was therefore decided to complement the steroid metabolism work with a study of the effects of 5α-reduced metabolites of testosterone on tumour growth.

The effects of the 5α-reduced products on the oestrous cycle and on plasma prolactin levels were also monitored. A recovery period was incorporated into the experimental design to examine the reversibility of any changes occurring during the treatment period. This meant that the effects of treatment on plasma oestradiol levels (which requires the collection of relatively large volumes of blood, obtainable only at sacrifice) could not be measured.

1. Experimental design

   (1) Tumours were induced as described in the METHODS section.

   (2) Rats bearing at least one actively growing tumour and exhibiting a regular cyclic pattern of vaginal smears were studied. Blood was withdrawn from the tail vein on the morning of dioestrus, and the animals were randomly allocated to one of 4 treatment groups (10 animals per group).

(3) The rats, 180 - 270g body weight, received 6 daily s.c. injections/ week of either vehicle, 5α-dihydrotestosterone, 5α-androstane-3α, 17β-diol or 5α-androstane-3β,17β-diol. The steroids were dissolved or suspended in corn oil containing 10% ethanol at a concentration of 5mg/ml and 0.2ml (= 1mg steroid), given as the daily injection.

(4) On the presentation of a dioestrus-type smear close to the 12th day after the commencement of treatment, treatment was stopped and a second sample of blood taken from the tail vein (i.e. 24 hours after the last injection of steroid or vehicle).

(5) No further treatment was given for approximately 12 days when another blood sample was taken from the tail vein on the morning of dioestrus. Tumours were excised, gross appearance noted and sections taken for histology.

Throughout the pretreatment (stage 1), treatment (stage 2) and recovery (stage 3) periods the oestrous cycle was assessed from daily vaginal smears and tumour measurements made 2 or 3 times per week. Blood samples were taken under ether anaesthesia between 1030 and 1200 hours and used for the measurement of plasma prolactin as described in the METHODS section. Tumours were discarded from the study if, after excision, they were not classified as adenocarcinoma or if necrotic tissue or pockets of fluid were found to contribute to the measurements of tumour size.

The lengths of treatment and recovery periods varied slightly, due mostly to the stipulation that these should end on a day when a positive dioestrus-type vaginal smear was observed. However, despite individual variation the average lengths of these periods were similar in all groups.

The results of this study are presented in Tables 31 - 35 and in Figs. 34 -39. Table 31 contains a summary of the individual results in Tables 32 - 35. Changes in tumour sizes in the different groups are diagrammatically represented by the

histograms in Figs. 34 - 35. Growth patterns of the tumours on individual representatives of each group are shown in Figs. 36 - 39.

2. Effect of androgens on the oestrous cycle

Immediately prior to allocation to a treatment group all animals exhibited regular patterns of vaginal smears. In the majority of cases the cycle lasted 4 days and, in others, 5 days.

In the control group, given vehicle only, 9 of the 10 rats continued to exhibit a cyclic pattern of vaginal smears throughout the treatment and recovery periods. The remaining rat showed a constant oestrus pattern of cornified cells in the vaginal smears for the latter half of the treatment period and throughout the recovery period.

Of the 10 rats which were given 5α-dihydrotestosterone, 6 presented a constant dioestrus pattern (many leukocytes present in vaginal smears) throughout the entire treatment period, 3 appeared to undergo one cycle before displaying constant dioestrus smears and the remaining rat cycled apparently normally throughout both the treatment and recovery periods. All of the 9 animals in this group which exhibited constant dioestrus patterns by the end of the treatment period remained in this state for 5 to 7 days into the recovery period after which the normal cyclic pattern was resumed.

Treatment with 5α-androstane-3α,17β-diol provided an essentially similar picture. Of the 10 animals in this group 6 displayed a constant dioestrus pattern throughout the treatment period and the remaining 4 appeared to undergo one cycle before the smear pattern was arrested in dioestrus. The pattern of constant dioestrus in the 5α-androstane-3α,17β-diol-treated group persisted for 2 to 9 days into the recovery period. The constant dioestrus pattern was replaced in 9 rats by a normal cyclic pattern and in one rat by a pattern of constant oestrus.

In contrast to 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol did not induce a constant dioestrus pattern of vaginal

Table 31

The effect of androgens on tumour growth - summary

Group	Stage	Plasma prolactin (ng/ml)	Oestrous cycle	Tumour size (cm^2)	Tumour growth					
					Total			Growing initially		
					G	S	R	G	S	R
Control	1	20.3	normal	1.75	16	7	0	16	0	0
(N = 10	2	19.5	normal	1.84	14	8	6[b]	8	5	3[b]
n = 29)	3	19.3	normal	2.27	15	7	7	8	4	4
5α-DHT	1	24.8	normal	1.78	22	10	2	22	0	0
(N = 10	2	20.1	persistent D	1.11	2	2	30[c]	2	2	18[c]
n = 34)	3	36.0	normal	1.45	12	8	10	8	7	7
5α-Adiol(3α)	1	26.1	normal	2.04	22	8	0	22	0	0
(N = 10	2	35.9	persistent D	1.19	1	7	22[c]	1	3	19[c]
n = 30)	3	60.5[a]	normal	1.27	7	8	14	6	6	10
5α-Adiol(3β)	1	51.1[a]	normal	2.21	16	6	0	16	0	0
(N = 10	2	29.5	normal	2.28	12	4	8[b]	9	3	4[b]
n = 26)	3	26.4	normal	2.70	13	4	8	8	3	4

N is the number of animals and n is the total number of tumours in each group.

a significantly different from the corresponding values of the control group and from the values of the other stages within the relevant group ($p < 0.05$ by variance analysis + Dunnett's test)

b significantly different from the respective pretreatment distributions ($p < 0.05$ by Fisher's exact test)

c significantly different from respective pretreatment distributions and the control treatment distributions ($p < 0.001$), and from the respective recovery distributions ($p < 0.05$ by Fisher's exact test)

G = grown, S = static, R = regressed

Table 32

The effect of androgens on tumour growth - control group

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
132L	11	11	cyclic	cyclic	13	20	19	RNk	0.64G	0.80G	1.00G
								RAx	1.26S	1.08R	0.88R
								RIn	1.56S	1.44S	0.80R
								RGr	2.21G	2.36S	1.82R
144L	11	14	cyclic	cyclic	20	13	30	RAx	3.96G	4.50G	7.82G
154L	11	11	cyclic	cyclic	26	15	13	RNk	0.36G	1.20G	2.10G
								RIn	2.70G	2.86S	3.04S
168L	16	5	cyclic	cyclic	31	22	13	LCh	1.08S	0.99S	0.99S
								RAx1	0.80S	0.36R	0.36S
								RAx2	3.80G	4.75G	4.75S
								RAx3	2.88G	2.08R	1.82R
								LGr	- -	2.34G	2.52G
169L	13	8	cyclic	cyclic	14	8	16	LNk1	1.40S	1.68G	1.82G
								LNk2	0.60G	0.60S	0.64S
								RCh	2.34S	1.92R	1.76R
								RAn	- -	0.48G	0.99G

Table 32 (continued)

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
185L	15	22	cyclic	cyclic	16	22	43	RNk	2.70G	2.86S	1.30R
								LAx	- -	- -	1.20G
								LGr	- -	1.82G	5.72G
								LAn	0.80G	0.88S	0.81S
271L	13	22	cyclic	cyclic	9	14	13	LNk1	1.92G	2.70G	3.78G
								LNk2	1.08G	0.80R	1.08G
								RNk	0.48S	0.48S	1.08G
290L	13	22	1 cycle and stuck in 0	stuck in 0	16	27	24	LAx	1.80G	1.20R	0.72R
298L	13	12	cyclic	cyclic	50	39	15	RNk1	0.25G	1.30G	3.00G
								RNk2	- -	0.80G	0.80S
								RAx	2.16G	2.52G	2.89G
300L	16	12	cyclic	cyclic	8	15	7	LNk	- -	1.95G	3.15G
								RNk	3.52G	5.20G	7.25G

Table 32 (continued)

Rat	Period (days)		Plasma prolactin (ng/ml)			Tumour size (cm^2) + growth		
Stage:	2	3	1	2	3	1	2	3
mean	13.2	13.9	20.3	19.5	19.3	1.75	1.84	2.27
n	10	10	10	10	10	23	28	29
mean of initial tumours (n = 23)						1.75	1.92	2.24
mean of tumour size/rat (n = 10)						4.03	5.16	6.47
grown (G) existing						16	9	14
grown (G) new							5	1
grown (G) total							14	15
static (S)						7	8	7
regressed (R) partially						0	6	7
regressed (R) totally							0	0
regressed (R) total							6	7

Table 33

The effect of androgens on tumour growth - 5α-dihydrotestosterone-treated group

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
43L	12	10	stuck in D	cyclic by day 5	11	6	5	RNk	0.48S	- R	- -
								LAn	3.20G	1.08R	1.10S
								RAn	0.80S	0.36R	- R
69L	12	16	1 cycle and stuck in D	cyclic by day 5	18	29	95	LNk	0.90R	- R	- -
								RNk	2.24G	1.30R	0.77R
								LLAx1	0.36S	- R	- -
								LAx2	3.06G	1.20R	1.08S
								RAx	1.43R	0.40R	0.80G
105L	13	8	stuck in D	cyclic by day 4	12	6	18	LGr	4.83G	6.44G	8.12G
116L	12	14	stuck in D	cyclic by day 7	52	22	17	RNk	1.82G	0.72R	0.63S
								LAx1	1.10G	0.48R	0.25R
								LAx2	0.64S	0.48R	- R
								RIn	1.10G	0.80R	- R
								RGr	0.36S	- R	- -
203L	12	15	1 cycle and stuck in D	cyclic by day 6	30	31	33	LNk1	1.65G	1.10R	0.48R
								LNk2	0.64G	0.25R	- R
								LAx	3.80G	1.30R	0.48R
								RIn	3.60G	3.00R	4.68G

Table 33 (continued)

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
209L	13	21	stuck in D	cyclic by day 5	16	19	36	RNk1	1.20G	0.88R	0.90S
								RNk2	0.96G	0.96S	1.30G
								LCh	1.40G	0.72R	1.40G
								RAx	2.08S	0.63S	0.99G
217L	13	21	stuck in D	cyclic by day 6	50	41	58	RNk1	1.30S	0.80R	1.82G
								RNk2	0.96G	0.25R	0.25S
								RGr	2.52G	1.04R	2.10G
219L	13	14	cyclic	cyclic	5	11	26	RNk	0.25G	- R	1.82G
								LCh	0.64G	0.72S	0.72S
								LAx1	1.96S	- R	1.10G
								LAx2	5.75G	1.17R	1.50G
								LAx3	0.25G	0.49G	1.44G
								LAn	1.68S	1.08R	0.90R
233L	11	19	stuck in D	cyclic by day 6	29	24	15	RAx	2.55G	0.96R	0.72R
234L	14	10	1 cycle and stuck in D	cyclic by day 7	25	12	57	RNk	2.88S	1.20R	1.30S
								LAx	2.25G	1.17R	1.17S

Table 33 (continued)

Rat	Period (days)		Plasma prolactin (ng/ml)			Tumour size (cm^2) + growth		
Stage:	2	3	1	2	3	1	2	3
mean	12.5	14.8	24.8	20.1	36.0	1.78	1.11	1.45
n	10	10	10	10	10	34	28	26
mean of initial tumours (n = 34)						1.78	0.91	1.11
mean of tumour size/rat (n = 10)						6.06	3.10	3.78
grown (G) existing						22	2	10
grown (G) new							0	2
static (S)						10	2	8
regressed (R) partially						2	24	6
regressed (R) totally							6	4
regressed (R)							30	10

Table 34

The effect of androgens on tumour growth -

5α-androstane-3α, 17β-diol-treated group

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
70L	12	19	1 cycle and stuck in D	cyclic by day 9	14	21	99	LAx1	0.90S	0.90S	1.00S
								LAx2	2.80G	1.80R	2.70G
								LAx3	0.56G	0.48S	0.48S
								RIn	0.80S	- R	- -
								LAn	2.38G	1.54R	- R
								RAn	2.85G	1.54R	0.88R
112L	13	11	1 cycle and stuck in D	cyclic by day 9	21	80	108	RNk	1.30G	0.48R	0.25R
								LCh	4.18S	2.04R	0.96R
								LAx1	1.30S	0.80R	- R
								LAx2	0.36S	0.36S	0.25R
								RAx1	1.40G	0.72R	- R
								RAx2	1.00S	0.36R	- R
								RGr	3.96G	1.50R	0.72R
113L	13	10	stuck in D	cyclic by day 3	7	34	39	LAx	2.70S	2.52S	3.06G
								RAx1	1.40G	1.30S	0.80R
								RAx2	5.72G	2.24R	1.80R
								LIn	2.52G	2.70S	2.24R
164L	13	16	stuck in D	cyclic by day 6	37	68	25	RTh	2.56G	0.64R	1.50G
								LIn	1.20G	0.36R	0.80G
								RIn	1.96G	0.25R	0.25S

Table 34 (continued)

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
208L	14	11	1 cycle and stuck in D	cyclic by day 2	8	15	16	LTh	1.43G	0.99R	0.88S
246L	14	11	1 cycle and stuck in D	cyclic by day 8	17	67	128	RNk1	2.08G	2.40G	2.70G
								RCh	2.24G	1.30R	1.30S
								RTh	1.68G	0.72R	0.36R
247L	11	12	stuck in D	cyclic by day 6	78	31	70	RTh1	3.52G	2.40R	2.28S
254L	14	23	1 cycle stuck in D	stuck in 0 from day 7	43	18	90	RCh	1.17G	0.25R	0.36G
								RAx	2.21G	0.90R	0.96S
276L	14	14	stuck in D	cyclic by day 5	26	19	16	RAx	1.43G	0.40R	- R
279L	11	12	stuck in D	cyclic by day 9	10	6	14	RNk	2.70G	1.87R	2.94G
								RAx	0.81S	0.80S	0.90S

Table 34 (continued)

Rat	Period (days)		Plasma prolactin (ng/ml)			Tumour size (cm^2) + growth		
Stage:	2	3	1	2	3	1	2	3
mean	12.9	13.9	26.1	35.9	60.5	2.04	1.19	1.27
n	10	10	10	10	10	30	29	24
mean of initial tumours (n = 30)						2.04	1.15	1.01
mean of tumour size/rat						6.11	3.46	3.04
grown (G) existing						22	1	7
grown (G) new							0	0
static (S)						8	7	8
regressed (R) partially						0	21	9
regressed (R) totally							1	5
							22	14

Table 35

The effect of androgens on tumour growth -

5α-androstane-3β, 17β-diol-treated group

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
71L	13	10	cyclic	cyclic	12	3	4	RNk	2.70G	1.95R	1.50R
87L	14	15	cyclic	cyclic	21	21	10	LCh	1.43G	1.80G	2.24G
								LAx	-	-	0.25G 0.70G
								RAx	0.81S	0.96G	1.10G
								RIn1	1.10S	0.64R	0.80G
								RIn2	3.23G	3.20S	3.04S
114L	14	19	cyclic	irregular	97	29	41	RAx	4.83G	4.80S	5.75G
								LGr	0.80G	- R	- -
								LAn	-	-	0.90G 0.25R
								RAn	1.26G	1.68G	2.52G
117L	15	12	cyclic	cyclic	22	7	35	LNk	0.99S	0.64R	- R
								LCh	2.38S	2.38S	2.38S
								LAx1	0.36G	0.63G	1.17G
								LAx2	3.04G	2.52R	2.21R
								LAx2	3.04G	2.52R	2.21R
								RTh	-	-	- - 0.60G

Table 35 (continued)

Rat	Period (days)		Vaginal smear pattern		Plasma prolactin (ng/ml)			Site	Tumour size (cm^2) + growth		
Stage:	2	3	2	3	1	2	3		1	2	3
292L	10	14	cyclic	cyclic	66	60	25	RAx	2.08G	2.38G	2.34S
301L	13	14	cyclic	cyclic	27	13	42	RNk1	0.88G	1.30G	1.12R
								RCh	0.88S	0.64R	0.42R
								RIn	0.80S	0.48R	0.36R
								LGr	4.42G	5.58G	4.74R
325L	13	8	cyclic	cyclic	17	10	42	LAx	6.75G	8.99G	14.44G
331L	10	11	cyclic	cyclic	105	97	27	LAx	1.96G	2.70G	2.70S
335L	11	18	cyclic	irregular	77	27	53	LAx	2.52G	2.52S	2.88G
356L	11	9	cyclic	cyclic	67	28	27	RAx	1.95G	0.99R	1.54G
								LGr	- -	- -	2.04G
								RGr	3.40G	4.40G	8.00G

Table 35 (continued)

Rat	Period (days)		Plasma prolactin (ng/ml)			Tumour size (cm^2) + growth		
Stage:	2	3	1	2	3	1	2	3
mean	12.4	13.0	51.1	29.5	26.4	2.21	2.28	2.70
n	10	10	10	10	10	22	23	24
mean of initial tumours (n = 22)						2.21	2.33	2.79
mean of tumour size/rat (n = 10)						4.86	5.23	6.23
grown (G) existing						16	10	11
grown (G) new							2	2
static (S)						6	4	4
regressed (R) partially						0	7	7
regressed (R) totally							1	1
							8	8

Regression analyses of tumour size in Tables 32 - 35

Parameter	n	Stages	Analysis	F-value	p
Control					
tumour size		1-2	linear regression	1.97 -	NS
(excluding new)	23	2-3	linear regression	4.64*	<0.05
		1-2-3	linear regression	4.77*	<0.05
			deviation	0.13 -	NS
tumour size		1-2	linear regression	7.19*	<0.05
(all)	29	2-3	linear regression	5.46*	<0.05
		1-2-3	linear regression	14.19*	<0.001
			deviation	0.06 -	NS
tumour size/rat		1-2	linear regression	8.20*	<0.05
	10	2-3	linear regression	6.84*	<0.05
		1-2-3	linear regression	17.19*	<0.001
			deviation	0.03 -	NS
5α-dihydrotestosterone					
tumour size		1-2	linear regression	24.88*	<0.001
(all)	34	2-3	linear regression	2.95 -	NS
		1-2-3	linear regression	13.98*	<0.001
			deviation	11.92*	<0.001
tumour size/rat		1-2	linear regression	17.24*	<0.01
	10	2-3	linear regression	1.80 -	NS
		1-2-3	linear regression	11.46*	<0.01
			deviation	9.77*	<0.01

Regression analyses of tumour sizes in Tables 32 – 35

Parameter	n	Stages	Analysis	F-value	p
5α-androstane-3α, 17β-diol					
tumour size		1-2	linear regression	33.84*	<0.001
(all)	30	2-3	linear regression	1.76 -	NS
		1-2-3	linear regression	42.57*	<0.001
			deviation	7.51*	<0.01
tumour size/rat		1-2	linear regression	15.26*	<0.01
	10	2-3	linear regression	0.78 -	NS
		1-2-3	linear regression	15.61*	<0.001
			deviation	2.75 -	NS
5α-androstane-3β, 17β-diol					
tumour size		1-2	linear regression	0.58 -	NS
(excluding new)	22	2-3	linear regression	2.18 -	NS
		1-2-3	linear regression	3.44 -	<0.10
			deviation	0.40 -	NS
tumour size		1-2	linear regression	1.15 -	NS
(all)	26	2-3	linear regression	3.32 -	<0.10
		1-2-3	linear regression	5.42*	<0.05
			deviation	0.53 -	NS
tumour size/rat		1-2	linear regression	1.95 -	NS
	10	2-3	linear regression	2.13 -	NS
		1-2-3	linear regression	4.45*	<0.05
			deviation	0.30 -	NS

smears, but instead appeared to permit the continuation of normal oestrous cycles throughout the treatment period. During the recovery period the cyclic pattern continued in all but 2 rats. Both of these rats exhibited a constant dioestrus pattern for the first part of the recovery period followed by a series of smears resembling those found in oestrus.

3. Effect of androgens on plasma prolactin levels

With the exception of animals whose vaginal smear pattern was described as stuck in oestrus or irregular, all blood samples were taken on a day on which the rats exhibited a vaginal smear characteristic of dioestrus. Frequently several rats had to be handled simultaneously and stress factors could not always be avoided.

Considerable ranges of prolactin values were obtained at certain stages. Since the variance differed markedly between sets of figures, a logarithmic transformation of data was performed before statistical analysis. When the prolactin values for the different stages of each group were compared only 2 differed significantly from all the others (Table 31). The pretreatment prolactin levels of the 5α-androstane-3β,17β-diol-treated group and the levels after recovery of the 5α-androstane 3α,17β-diol-treated group were significantly higher than the control values of the corresponding stages and significantly higher than the other 2 stages within their respective groups.

Neither of these elevations is readily explicable, especially that of the values obtained for the 5α-androstane-3β,17β-diol group prior to treatment at which stage all animals were untreated and were randomly allocated to each group. It is feasible that an uneven distribution of stress factors, leading to raised plasma prolactin levels at the time of blood sampling, could account for these differences.

At the end of the treatment period no differences in plasma prolactin levels were observed between steroid-treated and control rats. In the cycling rats of the control group samples were taken in dioestrus, a stage of the cycle in

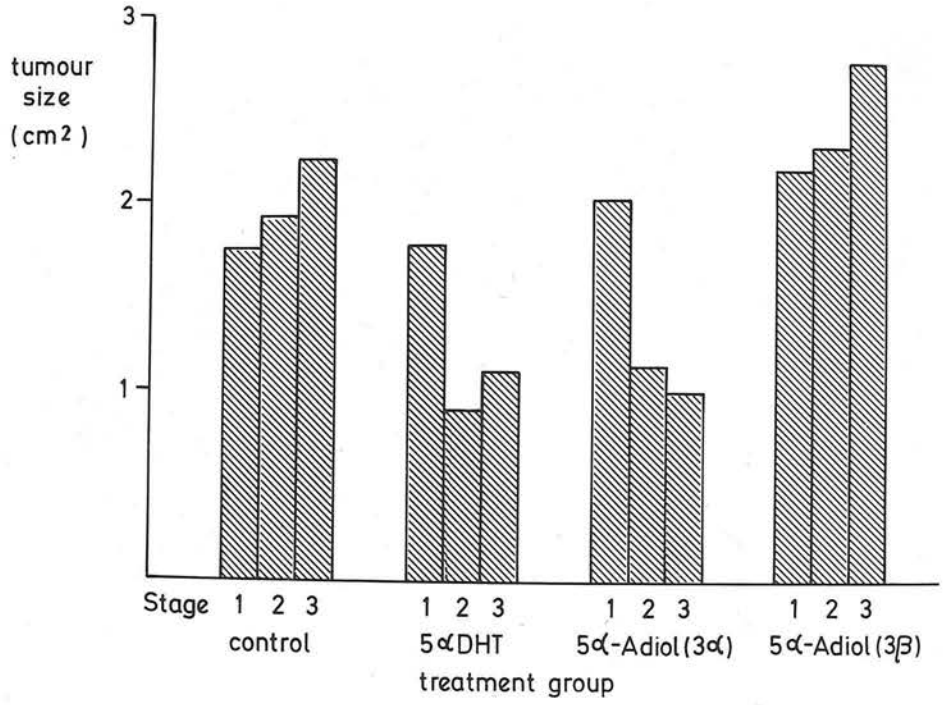
which plasma prolactin levels are relatively low. Therefore, the values obtained for the control group, and probably the 5α-androstane-3β,17β-diol-treated group, would not be representative of those throughout the treatment period during which far higher levels would have been attained at proestrus stages. On the other hand cyclic surges in plasma prolactin levels may not have occurred in the two groups showing constant dioestrus vaginal smears. The prolactin values obtained at the end of treatment in the 5α-dihydrotestosterone- and 5α-androstane-3α,17β-diol-treated groups were representative of the levels throughout the treatment period. As these were similar to the levels in dioestrus of cycling rats, it may be that by abolishing the cyclic surges of prolactin release, overall secretion was reduced by these 2 androgens.

4. Effect of androgens on tumour growth

From the regular measurements of tumour size it was possible to chart the growth pattern of the individual tumours as shown representatively in Figs. 36 - 39. Since the assessment of tumour size by caliper could not be accurately made below 0.25cm^2 (that is 0.5 x 0.5cm), only tumour sizes equal to or greater than these were recorded. The tumour sizes at the end of the pretreatment, treatment and recovery periods are given in the table along with a classification of growing rate into one of 3 broad categories: growing, static or regressing. Tumours showing changes in size of 10% or less over a 2 week period were classified as static. Changes in excess of 10% were classified as grown or regressed according to the direction of growth.

In control animals the initial tumour number was increased from 23 to 29 by the appearance of 5 new tumours in the treatment period and one in the recovery period. In the control group the highest proportion of growing tumours was seen in the pretreatment period whereas the proportion of tumours regressing was higher in the treatment and recovery period. A similar trend from growth to regression was also seen if one only considers tumours which were actively growing at the start of the treatment period (Table 32). This trend was

Figures 34 and 35. Effect of androgens on the size of DMBA-induced mammary tumours by mean tumour size and mean of the sum of tumour sizes/rat



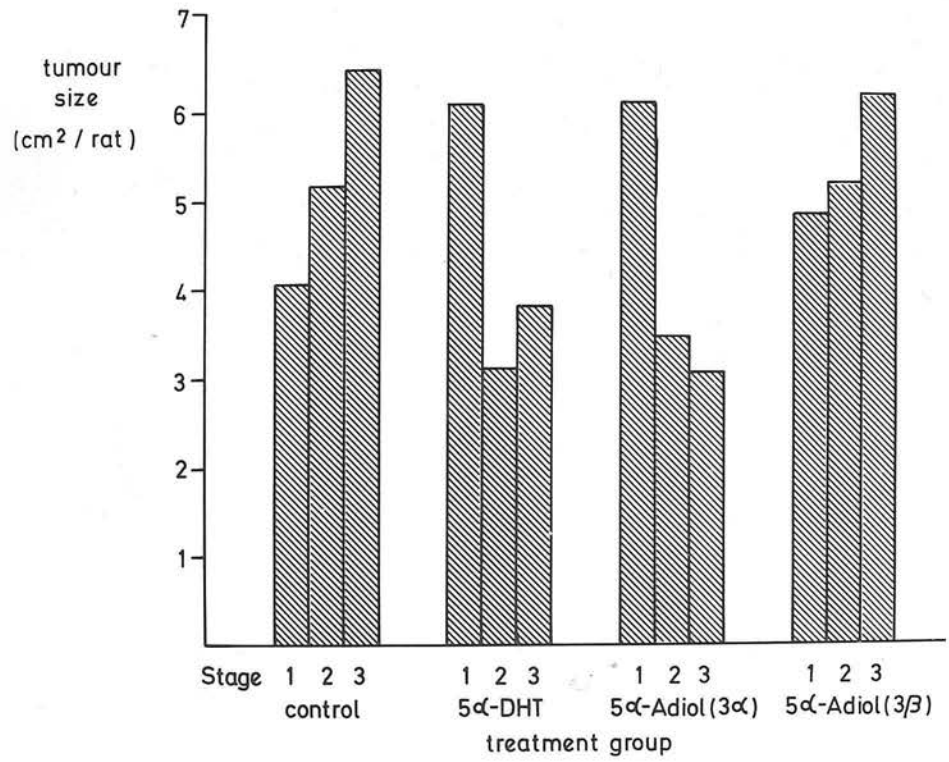
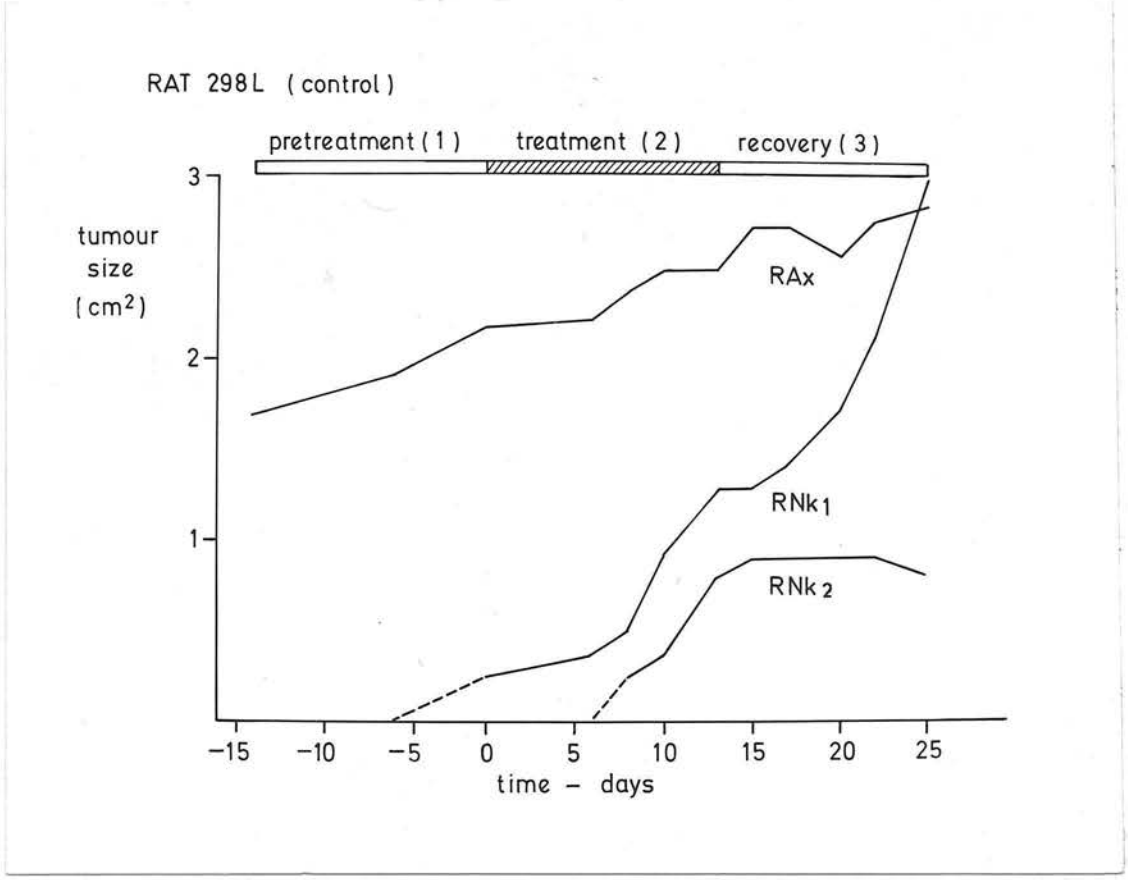


Figure 36. Effect of androgens on the growth of individual DMBA-induced mammary tumours

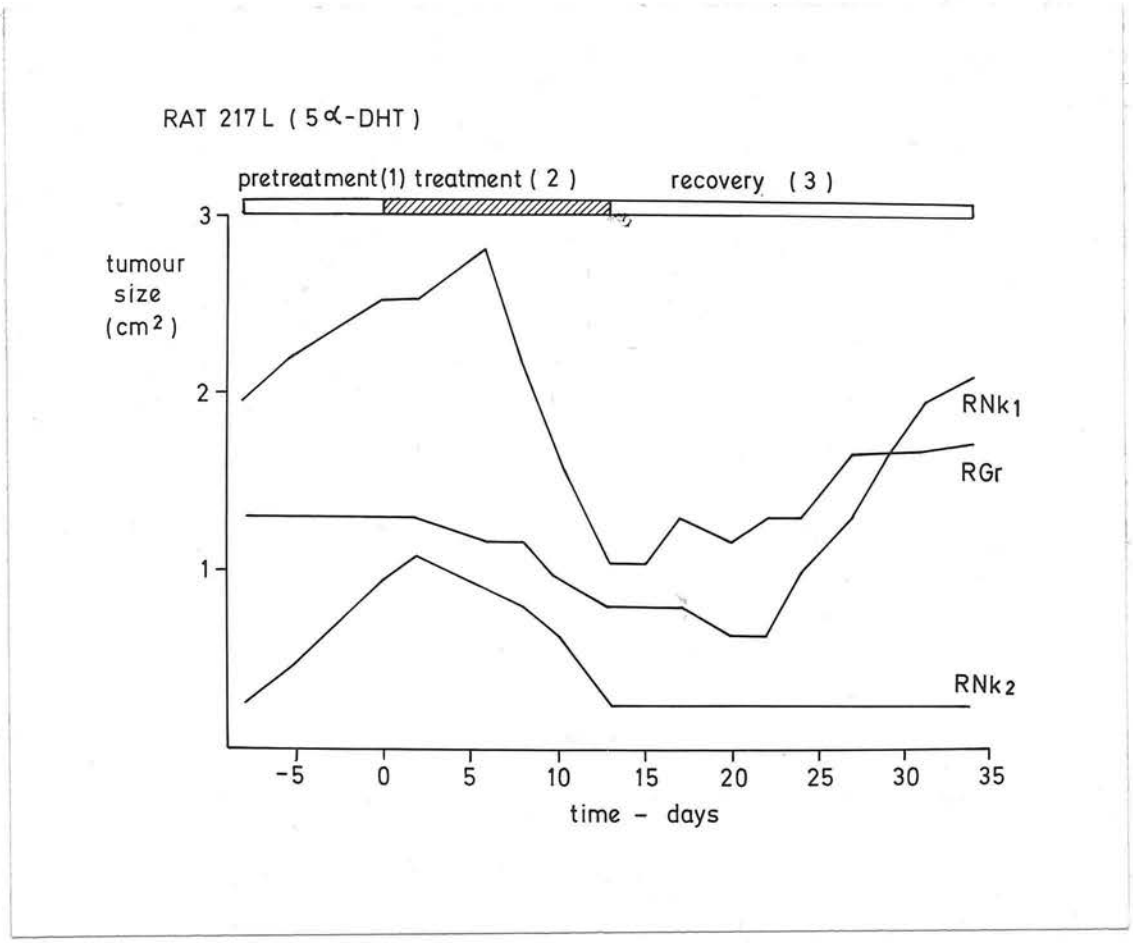
(a) Control



6 daily s.c. injections of corn oil (0.2ml) were given each week of the treatment period.

Figure 37. Effect of androgens on the growth of individual DMBA-induced mammary tumours

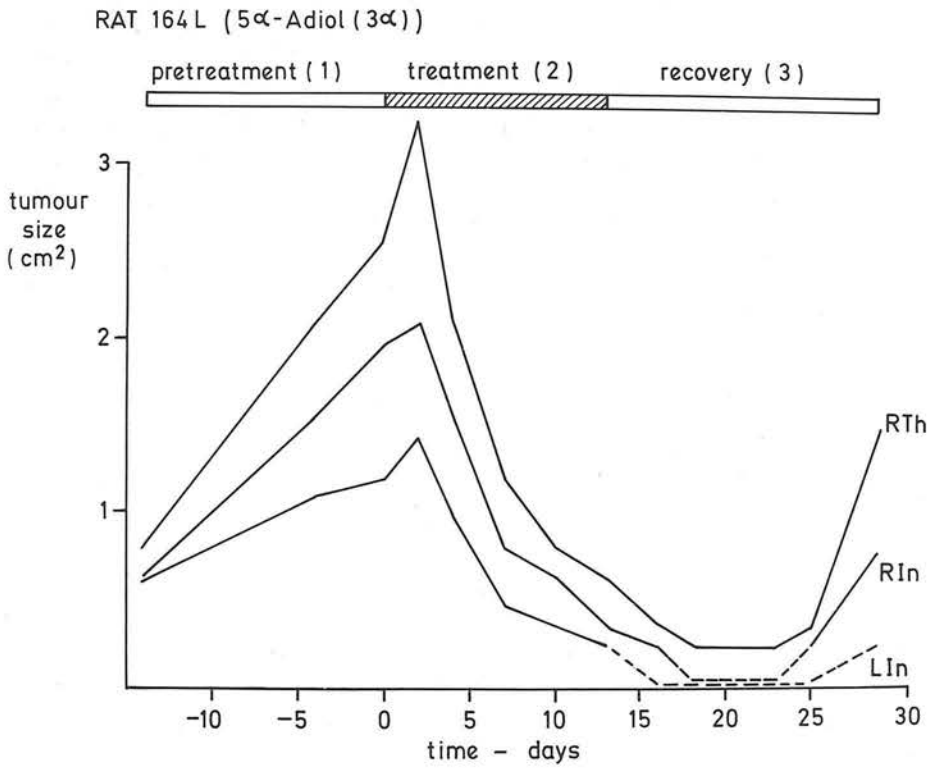
(b) 5α-dihydrotestosterone



6 daily s.c. injections of 5α-dihydrotestosterone (1mg) were given each week of the treatment period.

Figure 38. Effect of androgens on the growth of individual DMBA-induced mammary tumours

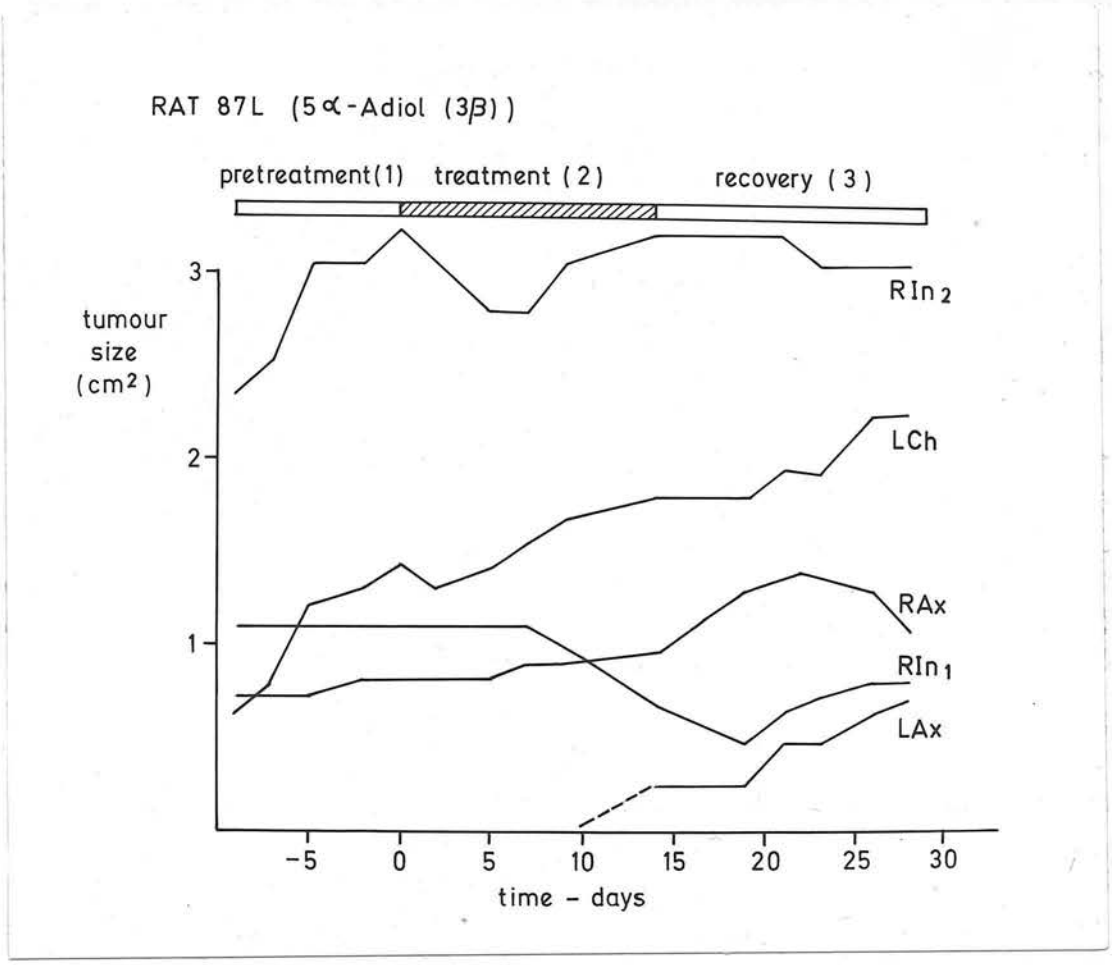
(c) 5α-androstane-3α, 17β-diol



6 daily s.c. injections of 5α-androstane-3α, 17β-diol (1mg) were given each week of the treatment period.

Figure 39. Effect of androgens on the growth of individual DMBA-induced mammary tumours

(d) 5α-androstane-3β, 17β-diol



6 daily s.c. injections of 5α-androstane-3β, 17β-diol (1mg) were given each week of the treatment period.

significant at the 5% level and emphasises the need for a control group in a study where static and spontaneously regressing tumours are encountered. The reason that less regressing tumours were seen in the pretreatment period is probably that animals were considered for use at an early stage of tumour growth and were allocated to the groups before extensive spontaneous regression became evident.

Despite this degree of spontaneous regression in the control rats treatment with both 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol could be seen to greatly increase the number of regressing tumours. After cessation of treatment the distribution of growing, static and regressing tumours in the 5α-dihydrotestosterone-treated group resembled that of the control group, but a less complete recovery was seen with 5α-androstane-3α,17β-diol. In the 5α-dihydrotestosterone-treated group 2 tumours appeared during the recovery period at sites where tumours had become impalpable during the treatment period. It was assumed for statistical analysis that these were regrowths of the original tumours although the possibility that they were completely new tumours cannot be discounted. This possibility excepted, no new tumours appeared in either the 5α-dihydrotestosterone- or 5α-androstane-3α,17β-diol-treated group throughout both treatment and recovery periods.

In the 5α-androstane-3β,17β-diol-treated group 4 new tumours appeared after the start of treatment, 2 in the treatment and 2 in the recovery period. However, in each of these periods a tumour regressed totally. No significant differences between the distributions of growing, static and regressing tumours of the control and 5α-androstane-3β,17β-diol-treated- groups were detected at any of the three stages.

The effect of the androgens was also assessed in terms of actual tumour size. This parameter was analysed by regression analysis which considers the extent of changes in tumour size without taking into account the absolute values. In the 5α-dihydrotestosterone- and 5α-androstane-3α,17β-diol-treated groups

in which no new tumours appeared, the number of tumours present initially could simply be used for the analysis of results. However, in the other 2 groups new tumours arose and there was a choice of considering only the tumours present at the start of the treatment or also including new tumours and ascribing to them a size just above zero (e.g. 0.01cm^2) in the periods before they became palpable. Both methods have drawbacks, the first obscures the possibility that the lack of new tumours in the 5α-dihydrotestosterone- and 5α-androstane-3α,17β-diol-treated animals may be regarded as a part of the effect of these steriods, and the second may introduce an arithmetical bias causing mean pretreatment values to be artifically lowered. The simplest solution was to perform analyses on both tumours present initially and on all tumours in the control and 5α- androstane-3β,17β-diol-treated groups. An alternative approach, which was applied to all 4 groups, was to sum the tumour size for each rat and use these values for analysis. It can be seen from the regression analyses and from the histograms (Figs. 34 and 35) that these different methods of analysis gave essentially the same picture.

In the control group the mean tumour size increased during both the treatment and recovery periods. Apart from the difference between pretreatment and treatment sizes for existing tumours only, all other differences were significant. The increase in tumour size appeared to be a function of time, with almost no deviation from linearity.

The administration of 5α-dihydrotestosterone caused a highly significant reduction in tumour size by the end of the treatment period. This regression in tumour size did not persist after the termination of treatment and the mean tumour size increased numerically, but not significantly, during the recovery period.

5α-androstane-3α,17β-diol likewise caused a highly significant decrease in tumour size. Tumour regression did not appear to continue after the cessation of treatment, and although regrowth of some tumours occurred, there was no increase in mean tumour size over this period.

Mean tumour size increased slightly during treatment with 5α-androstane -3β,17β-diol, and to a greater extent over the recovery period which might suggest a slight check in the growth rate of tumours by 5α-androstane-3β, 17β-diol. The general trend of increased tumour size over both treatment and recovery periods in this group was significant at the 5% level.

5. Summary of the effects of androgen treatment

Vehicle or 5α-androstane-3β,17β-diol appeared to have little effect on the oestrous cycle, whereas 5α-dihydrotestosterone or 5α-androstane-3α, 17β-diol arrested the oestrous cycle in dioestrus. The pattern of constant dioestrus persisted for approximately 6 days after the termination of treatment when it was replaced by the normal cyclic pattern. The plasma prolactin levels in these non-cycling rats at the end of the treatment period were similar to those found in the dioestrus stage of cycling control rats.

Both of the steroids which arrested the oestrous cycle also inhibited tumour growth and caused a profound reduction in tumour size. The regression of mammary tumours due to the administration of 5α- dihydrotestosterone and 5α-androstane-3α,17β-diol ceased after the termination of treatment, but there was frequently no regrowth of tumours during the recovery period. 5α-androstane-3β,17β-diol, which did not appear to affect the oestrous cycle, did not induce tumour regression.

VII. Influence of the stage of cycle and plasma prolactin levels at the time of DMBA administration on subsequent tumour development

1. Experimental design

The mammary tumours for this thesis were induced by a single intragastric administration of 30mg of DMBA in 2ml of cotton seed oil to female Sprague-Dawley rats, in groups of 5 - 25, at 47 - 55 days of age. Just prior to DMBA administration (1400 - 1600 hours), a sample of blood was taken for plasma prolactin assay and the stage of the oestrous cycle determined by a single vaginal smear. The animals were then palpated twice weekly for tumours for a period of 120 days after the administration of carcinogen. The number and size of all tumours were recorded. For the purposes of this study animals were divided into three separate categories:-

(a) Early death: Possibly, but perhaps not always, as a result of the DMBA administration some animals developed ill health (e.g. anaemia or respiratory problems), and either died or were put to death. The majority of these early deaths occurred or were inflicted within two weeks of the carcinogen administration. Since it could not be ascertained whether or not tumours would have developed in these animals the plasma samples were not assayed for prolactin content.

(b) Tumour-bearing: This group included all animals who, within 120 days of treatment, developed one or more mammary tumours of at least 0.8 x 0.8cm. This group was not further subdivided since many of the rats were allocated to treatment regimes or were sacrificed before the end of the observation period. Not all of the tumours were examined histologically, but the incidence of tumours other than adenocarcinoma has been shown to be very low in rats of this age (Gruenstein, Meranze, Thatcher & Shimkin, 1966).

(c) Non-tumour-bearing: All animals in this group were observed for at least 120 days after the administration of DMBA during which time any palpable lumps had not reached a size of 0.8 x 0.8 cm.

Vaginal smears were taken from a total of 861 rats at the time of DMBA administration. Of these, 170 (19.7%) died during the observation period or had to be killed due to ill-health. According to the above definitions, 459 (66.4%) of the remaining 691 rats developed tumours.

2. Stage of cycle and tumour incidence

A contingency table was constructed showing the frequency with which tumours occurred in rats at different stages of the cycle when given DMBA (Table 36). When the Chi-square test was applied to these data it was found that there was a significant difference between the distribution of tumour-bearing and non-tumour-bearing rats, over the four stages of the cycle. The source of this difference appears to lie with the two groups of rats which were in the proestrus and dioestrus stages of the cycle. Of the animals which were in proestrus on the day of DMBA administration, a greater proportion subsequently developed tumours, than in the other groups, whereas a lower than average incidence of tumours was found in animals which were in dioestrus on the day of DMBA administration.

In contrast there was no significant difference between the distribution of animals which suffered early death and the rest of the population with regard to the stage of cycle on the day of DMBA administration (Table 37). It can be seen from the totals in this second table that a far greater number of rats were classified as being in the oestrus or dioestrus stage of the cycle than in the other two stages. The uneven distribution probably reflects the difference in duration of each stage of the cycle (Long & Evans, 1922), and the fact that when the oestrous cycle is interrupted, vaginal smears of either a constant oestrus- or constant dioestrus-pattern are seen.

Table 36

Stage of cycle at DMBA administration

and subsequent incidence of tumours

	Stage of cycle when given DMBA								
	Proestrus		Oestrus		Metoestrus		Dioestrus		Totals
Tumours	103	(90.3)	171	(167.4)	50	(51.8)	135	(149.5)	459
No tumours	33	(45.7)	81	(84.6)	28	(26.2)	90	(75.5)	232
Totals	136		252		78		225		691

d.f. = 3, χ^2 = 9.9, therefore $p < 0.05$

Table 37

Stage of cycle at DMBA administration and subsequent health

	Stage of cycle when given DMBA								
	Proestrus		Oestrus		Metoestrus		Dioestrus		Totals
"Healthy"	136	(134.0)	252	(258.4)	78	(74.6)	225	(223.9)	691
Early death	31	(33.0)	70	(63.6)	15	(18.4)	54	(55.1)	170
Totals	167		322		93		279		861

d.f. = 3, χ^2 = 1.9, therefore no significant difference in frequency

Open figures refer to the number of rats in each category. Expected values are given in brackets.

3. Plasma prolactin and tumour incidence

The levels of prolactin in the plasma of blood samples removed just prior to DMBA administration varied from 2 to 380ng/ml with an arithmetic mean of 52.5ng/ml. The unsymmetrical continuous distribution of prolactin values appeared to be logarithmic-normal. Accordingly after transformation of the individual values to natural logarithms (ln), a symmetrical normal distribution was obtained. This transformation was used to compare the prolactin levels in the different categories in Table 38. Rats were divided into categories according to the stage of cycle at DMBA administration and the subsequent presence or absence of tumours.

It can be seen that at each stage of the cycle, the mean plasma prolactin level was lower in rats which subsequently bore no tumours compared to those which did. In proestrus and dioestrus, the difference between prolactin levels in tumour-bearing and non-tumour-bearing rats was significant, and in the other two stages of the cycle, the difference just failed to reach significance. When considered as a whole there was a highly significant systematic difference over all stages resulting in a p value of less than 0.001 when the plasma prolactin levels of all non-tumour-bearing rats were compared to those of all tumour-bearing rats.

In both tumour-bearing and non-tumour-bearing rats the plasma prolactin levels taken in oestrus were significantly higher than those taken in metoestrus and dioestrus.

4. Onset of the oestrous cycle

Since different strains of rats begin to cycle at different ages (Boyland & Sydnor, 1962), it was of interest to determine the onset of the oestrous cycle in relation to the time of DMBA administration in the rats used in this study. Therefore, a sample group of 9 rats was studied from an age of 30 to 56 days.

The vaginal plate in these rats opened between 32 and 36 days of age giving a constant dioestrus smear pattern for 5 - 7 days which was followed by

Table 38

Prolactin levels at DMBA administration

and subsequent tumour induction

	Proestrus	Oestrus	Metoestrus	Dioestrus	All
Tumours	3.72	3.88	3.55	3.61	3.73
	(41.4)	(48.5)	(34.7)	(36.9)	(41.7)
No tumours	3.28*	3.70	3.22	3.37*	3.45**
	(26.5)	(40.6)	(25.1)	(29.0)	(30.3)
All	3.61	3.82	3.43	3.51	3.64
	(36.9)	(45.7)	(30.9)	(33.5)	(38.1)

within group pooled s.d.'s: Tumours = 0.82, d.f. = 455; No tumours = 0.75, d.f. = 228

Open values are means of the ln prolactin concentration and the bracketed values below are the geometric means. The number of rats comprising each category are the same as in the corresponding cells in Table 36.

Variance analysis plus the F-test revealed a significant systematic difference between tumour-bearing and non-tumour-bearing rats across all stages of the cycle.

* and ** = significantly lower than corresponding values for tumour-bearing rats at $p < 0.05$ and $p < 0.001$ respectively.

Prolactin levels at oestrus were significantly higher than at metoestrus and dioestrus, $p < 0.05$ by the Student-Newman-Keuls test.

proestrus of the first cycle at 37 - 42 days of age. By 50 days of age, regular 4-day cycles were detected in 6 of the 9 rats, 2 appeared to undergo 5-day cycles and the remaining rat gave an irregular smear pattern. It can therefore be concluded that, in this study, the majority of rats were probably in the second or third oestrous cycle when they received DMBA.

5. Summary

Both the stage of the cycle and the level of prolactin at the time of DMBA administration appear to have affected the likelihood of an individual developing tumours over the following 120 days. Rats in proestrus at the time of DMBA administration had a slightly higher than normal tumour incidence, whereas the reverse was true for those in dioestrus. Irrespective of the cycle, tumour-bearing rats had higher mean levels of plasma prolactin at the time of DMBA administration, compared to non-tumour-bearing rats.

DISCUSSION

The presence of enzymes which metabolise steroids in a target tissue endow that tissue with the potential to control its local hormonal environment by the synthesis or inactivation of biologically active forms of steroids. In many androgen sensitive tissues it has been demonstrated that the expression of the androgenic effects of testosterone, the classical androgen, requires the presence of 5α-reductase to effect its conversion to the active form, 5α-dihydrotestosterone (King ε Mainwaring, 1974).

In common with certain human breast cancers, adenocarcinomas induced in the rat by the administration of DMBA appear to be androgen sensitive in that they regress on appropriate administration of androgens (Cooperative Breast Cancer Group, 1964; Griswold, Skipper, Laster, Wilcox ε Schabel, 1966). Both the human and rat mammary cancers are capable of converting testosterone by 5α-reduction to 5α-dihydrotestosterone (Miller, Forrest ε Hamilton, 1974). It was, therefore, proposed to measure the ability of DMBA-induced mammary tumours to metabolise testosterone by 5α-reduction, following alterations in the plasma levels of oestradiol and prolactin, the two hormones believed to be of greatest importance in controlling the growth of these tumours. An initial stimulus for this work was the finding that tumours induced and developed in rats rendered hyperprolactinaemic by perphenazine administration showed higher 5α-reductase activity than their control counterparts (Miller, 1976 a).

Hormonal control of enzyme activity can be studied in different systems. The effect of individual hormones can be studied in vitro by incubating portions of tissue with precursor in the presence and absence of hormone. The disadvantages of such a system are that the integral hormonal environment of the body, which may be required for the effect of an individual hormone to be observed, is lost, and that hormone-induced changes in enzyme activity, which require alterations in parameters such as protein or cell synthesis in vivo, will not be seen in short term in vitro incubations. The latter problem can be overcome by tissue or cell culture but the cell types surviving in these systems may not be representative of those in vivo and may show altered properties. A total in vitro approach also presents difficulties in relating hormonal effects on enzyme activity to those on the growth of the same tumour.

A second approach is to administer radioactively-labelled testosterone to hormonally manipulated animals, shortly before excising the tumour, which can then be studied for its content of radioactive steroid. This method is attractive because the effect of the hormonal manipulation on tumour growth can be assessed, and in situ measurement of metabolite formation should give a good reflection of the relative activities of tumour enzymes. However administration of the precursor and the tumour blood supply may vary and affect reproducibility, metabolites formed at other sites may enter the tumour before excision, and variations in steroid

levels within a tumour may reflect variations in accumulation and not variations in metabolism.

The approach adopted for the present study is a compromise of the two already described. Drug treatment and ovariectomy were used to alter the plasma levels of prolactin and oestradiol which were monitored, along with tumour size, before and during treatment. At the end of the treatment period tumours were excised and their capacity to metabolise testosterone assessed directly in vitro. Due to the complexity of hormonal interrelationships any single treatment probably alters the secretion of more than one hormone. It has therefore been necessary to integrate the data from different treatment regimes, in an attempt to determine which hormones may be involved in the control of 5α-reductase activity in the tumours.

All incubations of tumour have been performed under fixed, standard conditions so that a comparison of the capacity of tumour to metabolise testosterone by 5α-reduction could be made. A whole tissue homogenate of 0.5 g of tumour was incubated for one hour, with 50×10^6 d.p.m. testosterone in 0.5 ml Krebs-Ringer phosphate buffer, pH 7.4, containing an NADPH-generating system.

5α-Reductase activity has been found in both nuclear and the crude cytoplasmic fractions of the ventral prostate of rats (Baulieu ε Robel, 1970; Frederiksen ε Wilson, 1971), and of dogs (McGuire, Hollis ε Tomkins, 1960), and rat hypothalamus (Cheng ε Karavolas, 1975). It has recently been shown that 5α-reductase activity is

distributed almost equally in nuclear, mitochondrial and microsomal fractions of DMBA-induced rat mammary tumours (Mori, Tominaga ε Tamaoki, 1978; Tamaoki, Mori, Kitamura ε Tominaga, 1978). The 5α-reductase activity measured in the homogenates of whole tissue is therefore probably due to the action of enzymes located in different cell organelles. Although a measurement of overall 5α-reductase activity is probably most suitable for preliminary investigations such as these, it is possible that 5α-reductase activity of the various organelles could differ in their sensitivity to hormonal modulation.

Estimations of the optimum pH for 5α-reductase activity vary from about 6.6 to 7.0 depending on the system under study (Frederiksen ε Wilson, 1971; Koninckx, Verhoeven, Heyns ε de Moor, 1979). However the activity at pH 7.4 in these systems was still close to the maximum. If 5α-reductase of DMBA-induced mammary tumours exhibits a similar pH dependence, then 5α-reductase activity measured at pH 7.4, in this and other studies (King, Gordon ε Helfenstein, 1964; Miller, Forrest ε Hamilton, 1974; Mori, Tominaga ε Tamaoki, 1978), may be marginally below the maximum. This should not affect the intercomparison of tumour 5α-reductase capacity, since, assuming that pH dependence is alike in all tumours, the relative activities of 5α-reductase will always be the same provided that a fixed pH is used. The optimum temperature for 5α-reductase activity in the rat ventral prostate preparation was found to be 37°C (Bruchovsky ε Wilson, 1968; Frederiksen ε Wilson, 1971).

The concentration of testosterone precursor present at the start of the incubation was approximately 100 ng/ml (= 3.5×10^{-7}M), which is almost 500 times greater than the highest concentration of testosterone in blood of female rats during the oestrous cycle (Dupon ε Kim, 1973; Weizenbaum, Adler ε Ganjam, 1979). The concentration of the $NADP^+$ for the generation of NADPH by the reaction of added glucose-6-phosphate with glucose-6-phosphate dehydrogenase was 2×10^{-4}M. These features, in combination with loss of both tissue and cellular spatial integrity during the homogenisation procedure, create an artificial system. It cannot therefore be assumed that the pattern of metabolism observed under these conditions will reflect the situation existing in vivo. However it is hoped this system will measure the relative capacity of tumours to perform the 5α-reduction of testosterone.

The observation that 5α-reductase activity varied far less between duplicate portions of the same tumour than between tumours of the same rat or tumours of different rats indicated the reproducibility of the method and the validity of its use comparing the capacity of different tumours to perform 5α-reduction. The variations observed within tumours may have arisen from methodological limitations or from regional differences in enzyme activity within the tumour. The latter possibility might occur if 5α-reductase levels were higher in a particular cell type since histological variations are sometimes observed within individual tumours (Huggins,

Briziarelli ε Sutton, 1959; Young ε Hallowes, 1973). It has been claimed that testosterone 5α-reductase is located predominantly in the stroma, with very little in epithelium of benign hyperplastic human prostate (Cowan, Cowan ε Grant, 1976).

The quantities of testosterone metabolised by DMBA-induced mammary tumours obtained from untreated rats, and incubated under the standard conditions, varied considerably. On average approximately half of the precursor was metabolised after a one hour incubation period. The majority of this metabolism was accounted for by 5α-reduction which was measured as the sum of the net production of 5α-dihydrotestosterone and 5α-androstanediol. The production of 5α-androstanedione was so low, that it was decided not to measure its formation for inclusion in the estimation of 5α-reduction. The 1.03% conversion of testosterone to 5α-androstanedione, after 60 minutes in a time-course experiment, probably represents the top end of the range of its production since a small, but distinct, radioactive peak, associated with 5α-androstanedione, was observed on scanning the initial plate of that incubation, whereas the corresponding peaks were either smaller or absent on other initial plates.

Although the 3α,17β-isomer of 5α-androstanediol was added as a carrier steroid the methods employed give a measure of total 5α-androstanediol production, since all four isomers of 5α-androstanediol migrate together in the initial solvent system and subsequent oxidation

yields a common product, 5α-androstanedione (Miller, 1976 b). When the 3α,17β- and 3β,17β-isomers of 5α-androstanediol were separated in solvent system V before oxidation, it was found that 3α,17β-isomer accounted for most of the 5α-androstanediol production. Although the 17α-isomers were not separately studied, it is possible that the 3β,17β-isomer could have been contaminated with the 3β,17α-isomer which has a similar mobility in solvent system V (Berthou, Bardou ε Floch, 1974). However there are no reports of the formation of this latter steroid by the DMBA-induced mammary tumour. Contamination by the 3α,17α-isomer is highly unlikely since it migrates more slowly than the other three isomers in solvent system V (Berthou, Bardou ε Floch, 1974).

An interesting pattern of net production of 5α-reduced metabolites with time was seen. Over about the first 10 minutes, the net production rate of 5α-dihydrotestosterone exceeded that of 5α-androstane-3α,17β-diol, but between 10 and 60 minutes this situation was reversed giving the greatest ratio of 5α-androstanediol to 5α-dihydrotestosterone at 60 minutes. However by 120 minutes the levels of 5α-dihydrotestosterone in the incubation again exceeded those of 5α-androstane-3α,17β-diol, the latter actually decreasing during this period. In contrast the production of 5α-androstane-3β,17β-diol continued to rise throughout the entire incubation period. The overall 5α-reduction rate almost mirrored the decrease in precursor level, both proceeding rapidly over the first hour and then becoming markedly slower.

The pattern of metabolism can be interpreted as an irreversible conversion of testosterone to 5α-dihydrotestosterone which initially exceeds the further metabolism of 5α-dihydrotestosterone to 5α-androstanediols. The 5α-dihydrotestosterone so formed can either be reversibly converted to 5α-androstane-3α,17β-diol by the highly active 3α-hydroxysteroiddehydrogenase or irreversibly converted to 5α-androstane-3β,17β-diol by the less active 3β-hydroxysteroiddehydrogenase. The rapid conversion of 5α-androstane-3α,17β-diol but not its 3β-isomer to 5α-dihydrotestosterone has also been demonstrated in other tissues such as the rat prostate (Baulieu, Le Goascogne, Groyer, Feyel-Cabanes ε Robel, 1975), and rat anterior pituitary (Pilven, Thieulant, Ducouret, Sampérez ε Jouan, 1976). From the results of the time-course experiments it appears that the DMBA-induced mammary tumour has a similar pattern of metabolism. This is schematically shown on Fig. 40.

Although the fact that the net production of 5α-dihydrotestosterone between 60 and 120 minutes was greater than the total metabolism of testosterone over the same period can only be explained by the reverse conversion of 5α-androstane-3α,17β-diol to 5α-dihydrotestosterone, it is not readily apparent why the overall direction of the reaction should have changed. When the metabolism of testosterone by the nuclear fraction of rat prostate was measured in the presence of varying concentrations of $NADP^+$ it was found that although 5α-reduction remained constant at concentrations

greater than 10^{-5}M, this was accounted for mainly by the formation of 5α-dihydrotestosterone at lower concentrations of $NADP^+$ but at higher concentrations 5α-androstane-3α,17β-diol was the major metabolite formed (Frederiksen ε Wilson, 1971). It is therefore possible that in the present study the high initial concentration of $NADP^+$ favoured the conversion of 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol, but that as $NADP^+$ was converted to NADPH the reverse reaction became favoured. This explanation is further supported by the observation that in the time-course incubation performed without the addition of $NADP^+$ or the other components of the NADPH-generating system, 5α-androstane-3α,17β-diol formation was much lower than that of 5α-dihydrotestosterone and there was no indication of a reverse conversion of the diol to 5α-dihydrotestosterone.

The omission of all components of the NADPH-generating system profoundly reduced the initial rate of 5α-reduction of testosterone by DMBA-induced mammary tumours, and also the further conversion of 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol, indicating the necessity of adding such a system to measure full 5α-reductase capacity in a standard 60 minute incubation period. However it was remarkable that 5α-reductase activity increased to such an extent during the course of the incubation that the formation of 5α-reduced products had reached the same level as that of the standard incubation within 120 minutes. Since it has been widely demonstrated that 5α-reductase in subcellular fractions

has an absolute requirement for NADPH (Wilson ε Gloyna, 1970; Frederiksen ε Wilson, 1971), it would appear that the DMBA-induced tumour is capable of producing a substantial quantity of NADPH. Indeed, the pentose-phosphate pathway and the presence of glucose-6-phosphate dehydrogenase, NADP-isocitrate dehydrogenase and NADP-malate dehydrogenase, which facilitate NADPH production, have all been demonstrated in the DMBA-induced tumour (Hilf, Goldenberg, Gruenstein, Meranze ε Shimkin, 1970; Cohen ε Hilf, 1974). It is possible, of course, that 5α-reductase of the DMBA-induced mammary tumour can utilise another co-factor such as NADH.

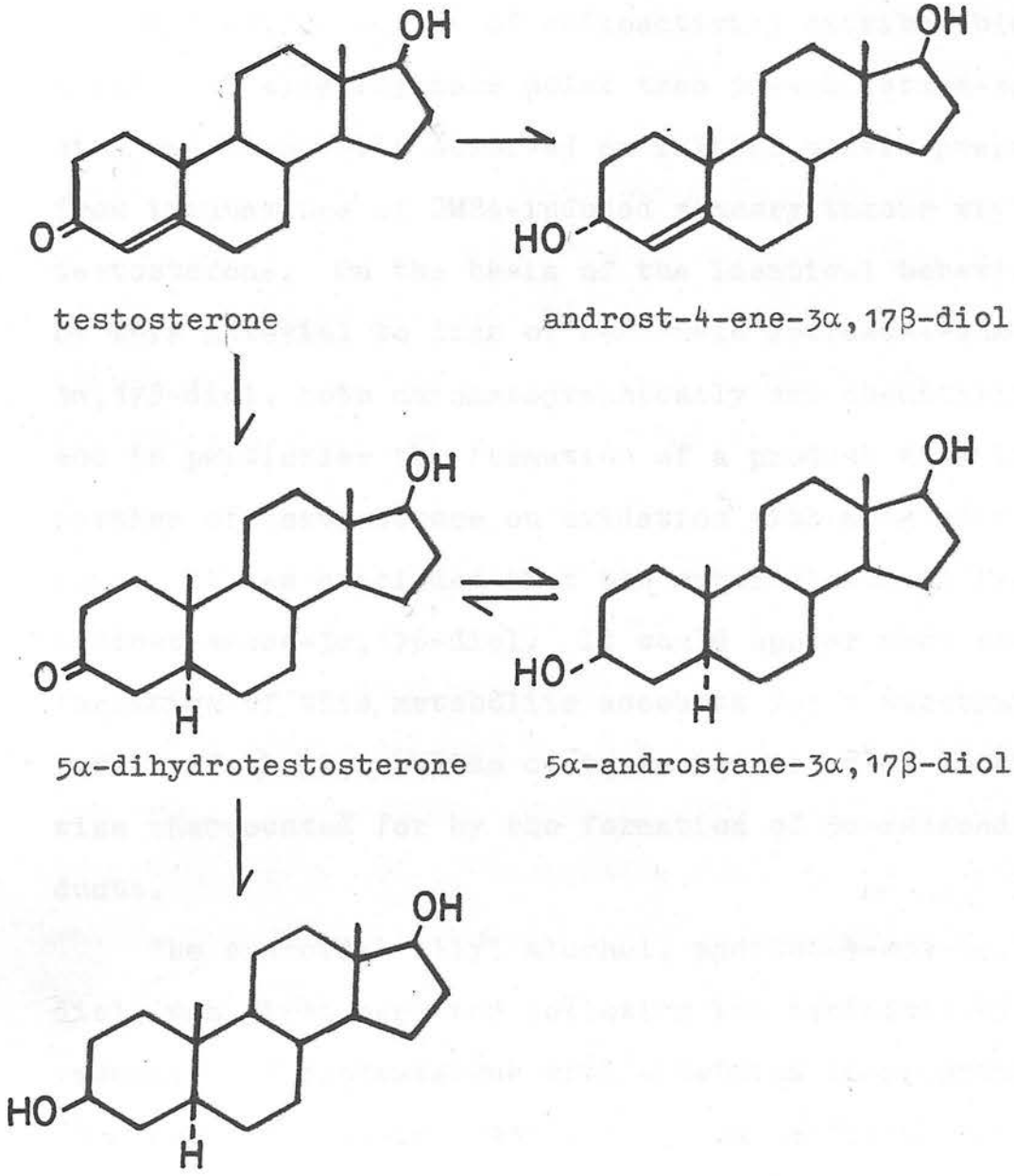
A cessation of 5α-reductase activity in preparations where living cells are not maintained has been reported to occur within 100-120 minutes at 37°C (Frederiksen ε Wilson, 1971). On the basis of other experimental data, it was concluded that the effect was not due to lack of co-factors nor product inhibition of the enzyme, but to the thermal denaturation of the enzyme at 37°C. Although the rate of 5α-reduction between 60-120 minutes was slower than in the first hour, under the standard incubation of the present study, there was evidence of high 5α-reductase activity between 60-120 minutes in the incubation without an added NADPH-generating system. This latter observation would suggest that the enzyme is not so labile in the whole tissue homogenate of DMBA-induced mammary tumours. This could be due to the presence of some whole cells in the preparation. In any case, apart from the time-course studies, an incubation

period of 60 minutes was always used.

In the light of observations mentioned above, it is interesting to compare the results of the present study with those of other workers in this field. In all preparations of the DMBA-induced mammary tumours so far studied 5α-reductase has been identified as the major route of testosterone metabolism (King, Gordon ε Helfenstein, 1964; Miller, Forrest ε Hamilton, 1974; Mori, Tominaga ε Tamaoki, 1978; Abul-Hajj, 1979). Some of these workers have found that the levels of 5α-dihydrotestosterone present at the end of the incubation period were greater than those of 5α-androstanediol (King, Gordon ε Helfenstein, 1964; Miller, Forrest ε Hamilton, 1974; Miller, 1976 a), whereas 5α-androstanediol was always found in greater quantities than 5α-dihydrotestosterone at the end of the standard incubation used in the present study. From the observations made during the time-course studies, the probable explanation for this difference is the use of a two-hour incubation period in all of these studies, compared with a one-hour period in the present study and also the lack of an NADPH supply in the earlier study of King, Gordon and Helfenstein (1964). This explanation is further supported by reports of the recovery of more 5α-androstanediol than 5α-dihydrotestosterone after incubation of one hour in the presence of NADPH (Miller, 1976 b; Mori, Tominaga ε Tamaoki, 1978).

To my knowledge there has been no positive identification of the formation of androst-4-ene-3α,17β-diol

Figure 40. Additions to metabolism of C-19 steroids by DMBA-induced mammary tumours based on results from the present study



by DMBA-induced rat mammary tumours. In this thesis, evidence is presented which suggests that a considerable amount of androst-4-ene-3α,17β-diol can be formed from testosterone by these tumours.

A distinct region of radioactivity attributable to a material slightly more polar than 5α-androstane-3α,17β-diol was frequently observed on initial plates prepared from incubations of DMBA-induced mammary tumour with testosterone. On the basis of the identical behaviour of this material to that of authentic androst-4-ene-3α,17β-diol, both chromatographically and chemically, and in particular the formation of a product with the properties of testosterone on oxidation with manganese dioxide, it was concluded that the material was in fact androst-4-ene-3α,17β-diol. It would appear that the formation of this metabolite accounts for a substantial amount of the metabolism of testosterone which is otherwise unaccounted for by the formation of 5α-reduced products.

The steroidal allyl alcohol, androst-4-ene-3α,17β-diol, was first reported following its synthesis by the reduction of testosterone with aluminium isopropoxide (Butenandt ε Heusner, 1938), but, presumably because chemical reduction of testosterone yields very little 3α-isomer (Jackman, Killen-Macbeth & Mills, 1949; Killen-Macbeth ε Mills, 1949; Berthou, Bardou ε Floch, 1974), it is not available commercially and has received little attention.

Evidence has been presented for the formation of steroidal allyl alcohols by preparations of ovary and adrenal (Levy, Saito, Takeyama, Merrill ε Schepis, 1963), liver (Lisboa ε Gustafsson, 1970; Sanyal, Orr ε Engel, 1974), uterus (Lisboa ε Holtermann, 1976; Junkerman, Runnebaum ε Lisboa, 1977), and normal breast of mouse (Mori ε Tamaoki, 1977) and rat (Tamaoki, Mori, Kitamura, ε Tominaga, 1978; Mori, Tominaga ε Tamaoki, 1978). Smith and King (1966) found no evidence for the formation of androst-4-ene-3α,17β-diol from testosterone by normal mammary glands and the spontaneous pregnancy-dependent mammary tumours of BR6 mice, but were able to demonstrate its production by all pregnancy-independent tumours investigated. They concluded that the differences found between the two types of tumour could have been due to differences in the rate of either the synthesis or degradation of androst-4-ene-3α,17β-diol. In two recent publications (Tamaoki, Mori, Kitamura ε Tominaga, 1978; Mori, Tominaga ε Tamaoki, 1978), it was reported that although androst-4-ene-3α,17β-diol was the only metabolite of testosterone found in the normal rat mammary gland and was produced in substantial quantities, there was no evidence of its formation by the DMBA-induced rat mammary tumour. Since DMBA-induced mammary tumours are encapsulated and in the present study were removed in entirety before taking a portion from the centre, free of capsule, for metabolism studies, it is highly unlikely that the divergence in findings is due to contamination of the tumour with normal breast

tissue. However it is possible that the extent of androst-4-ene-3α,17β-diol formation is related to the presence of non-cancerous cells within the tumour and may give an index of the differentiation of tumours. The enzyme which catalysed the conversion of testosterone to androst-4-ene-3α,17β-diol in the normal mammary gland was found to reside in the cytoplasmic fraction (supernatant of homogenates centrifuged at 105,000 g for 60 minutes), and had a preference for NADPH to NADH as co-factor. It was not clear whether this enzyme was the same as that which catalysed the reduction of the 3-keto group of 5α-reduced steroids (Mori, Tominaga ε Tamaoki, 1978).

Lisboa and Gustafsson (1970) found that the formation of androst-4-ene-3α,17β-diol was inversely related to the formation of 5α-reduced products and concluded that the allyl alcohol was only formed when 5α-reductase activity was extremely low. The same inverse correlation was found in the present study, but the formation of androst-4-ene-3α,17β-diol still occurred when 5α-reductase activity was relatively high. Smith and King (1966) postulated that the further conversion of androst-4-ene-3α,17β-diol to 5α-androstane-3α,17β-diol was unlikely as 4-ene-5α-steroiddehydrogenase in other tissues does not act in the presence of a 3-hydroxyl group (Rongone, 1962; Breuer, Dahn ε Norymberski, 1963). However the formation of 4-androstenedione and testosterone from androst-4-ene-3α,17β-diol by chick liver has been reported (Ungar, Gut ε Dorfman, 1957; Sanyal, Orr ε Engel, 1974). Since no indications as to the

further metabolism of androst-4-ene-3α,17β-diol were obtained in the present studies, only its formation from testosterone is indicated in Fig. 40.

The only relevant publication on the biological activity of androst-4-ene-3α,17β-diol which could be found was that of Huggins and Mainzer (1957), in which it was reported that the daily administration of 0.5 mg of the steroid was as effective as ovariectomy in causing a regression of transplantable hormone-dependent mammary tumours. Whilst it is possible that this activity was mediated via the conversion of androst-4-ene-3α,17β-diol to testosterone the fact that it may be an inhibitor of mammary tumour growth, and is formed from testosterone as an alternative to 5α-reduction by DMBA-induced tumours, makes it worthy of further study. This may be facilitated by a recent report of a new and easier method of synthesis (Mori, Ikegami ε Tamaoki, 1979).

The assessment of plasma prolactin levels is hampered by the fact that the secretion of prolactin by the pituitary is rapidly raised by stressful situations, such as exposure to ether (Wuttke ε Meites, 1970; Wakabayashi, Arimura ε Schally, 1971; Stern ε Voogt, 1973). The two procedures which circumvent this problem, decapitation without anaesthetic and withdrawal of blood through an indwelling cannula in the conscious animal, were not suited to the present studies which frequently required presacrifice sampling and which involved such large numbers of animals as to make the implantation of cannulae impractical. With practice it

was possible to complete the collection of blood from the tail vein of every rat within 120 seconds of its removal from the stable environment of the animal room, and within 90 seconds of its initial exposure to ether. It has been shown that plasma levels of prolactin are significantly raised two minutes after brief exposure to ether and remain elevated for about 15 minutes (Krulich, Hefco, Illner ε Read, 1974; Simonel, Brooks ε Welsch, 1975; Mattheij ε van Pijkeren, 1977). Whilst it is questionable whether the method employed in the present studies will have been rapid enough to avoid stress-induced effects on plasma prolactin, several workers have demonstrated that, in female rats, the release mechanism for prolactin appears to be relatively insensitive to stress in dioestrus, the stage of the cycle in which most plasma prolactin measurements have been made (Neill, 1970; Riegle ε Meites, 1976; Mattheij, Kuipers, Sterrenberg ε Swarts, 1979).

Although not ideal, the method employed was satisfactory in that plasma prolactin levels were found to be significantly altered in the expected directions by the various treatments employed. It is likely that the relatively higher levels of plasma prolactin found at the oestrus stage of the cycle in rats at the time of DMBA administration resulted from stress-induced effects. Although Pearson, Llerena, Llerena, Molina and Butler (1969) also found prolactin levels higher in the oestrus stage of the cycle, according to Neill (1970 ε 1972), prolactin levels are only elevated in proestrus and not in oestrus in unstressed rats.

The secretion of prolactin from the anterior pituitary is largely controlled by prolactin inhibitory factor which is released from the hypothalamus into the hypothalamic-hypophysial portal system (Meites, Nicoll ε Talwalker, 1963; Meites ε Nicoll, 1966). Recent evidence has indicated that PIF is probably dopamine and that oestradiol, which, in the normal cycling rat, is the main physiological stimulus for prolactin release, acts both by inhibiting PIF release from the hypothalamus and by directly blocking the action of PIF at pituitary level (Labrie, Borgeat, Drouin, Beaulieu, Lagacé, Ferland ε Raymond, 1979). Thus plasma levels of prolactin are dramatically elevated by the surge of oestradiol at proestrus and fall sharply on ovariectomy (Pearson, Llerena, Llerena, Molina ε Butler, 1969; Amenomori, Chen ε Meites, 1970; Chen ε Meites, 1970; Neill, Freeman ε Tillson, 1971).

Perphenazine, a phenothiazine, has been shown to powerfully stimulate prolactin secretion (Pearson, Llerena, Llerena, Molina ε Butler, 1969; Ben-David, Danon ε Sulman, 1970). Like oestradiol, perphenazine may act at a hypothalamic level (Danon ε Sulman, 1970), or directly on the pituitary (Shani, Givant, Sulman, Eylath ε Eckstein, 1971; MacLeod ε Lehmeyer, 1974). In the present study it was found that elevations in prolactin levels could not be reproducibly demonstrated in plasma collected 24 hours after the last of a series of daily injections of a commercial aqueous preparation of perphenazine. This result was perhaps not surprising,

since it has previously been shown that 24 hours after a single injection of a similar preparation plasma prolactin levels had returned almost to pre-injection values (Pearson, Llerena, Llerena, Molina ε Butler, 1969; Ben-David, Danon ε Sulman, 1970).

When corn oil was used as a vehicle for perphenazine a single s.c. injection of this preparation induced almost as rapid a rise in plasma prolactin levels as that reported for the aqueous preparations. However plasma prolactin levels were still significantly raised 24 hours after the injection of perphenazine in corn oil. The longer lasting effect of the corn oil preparation is probably due to a slower release of perphenazine from the corn oil injection depot. Using corn oil as a vehicle, elevated plasma prolactin levels were also found 24 hours after the last of a series of daily injections of perphenazine. In agreement with others perphenazine was found to be equally effective in raising plasma prolactin levels in intact and ovariectomised rats (Pearson, Llerena, Llerena, Molina ε Butler, 1969). Although plasma prolactin levels were clearly elevated by perphenazine two points should be borne in mind when comparing levels in intact control and perphenazine-treated rats. The first is that peak plasma prolactin levels in perphenazine-treated rats were probably reached in less than 24 hours and the second is that intact control rats were always killed in dioestrus when plasma prolactin levels are much lower than those of proestrus (Butcher, Collins ε Fugo, 1974).

In common with other ergot alkaloids, CB 154 has been shown to inhibit prolactin secretion mainly by acting as a potent dopamine agonist at pituitary level (Zeilmaker ε Carlsen, 1962; Pasteels ε Ectors, 1970; Lu, Koch ε Meites, 1971). When administered in corn oil, CB 154 caused a profound reduction in plasma prolactin levels which lasted over 24 hours.

The low levels of plasma oestradiol found at oestrus and metoestrus and the high levels found at proestrus in intact untreated rats in the present study correspond to those reported in more detailed studies (Brown-Grant, Exley ε Naftolin, 1970; Butcher, Collins ε Fugo, 1974). Oestradiol levels at dioestrus showed some variation and were on average above those of oestrus and metoestrus. The degree of variation is probably related to the gradual rise in oestradiol levels seen throughout the dioestrus stage of the cycle (Brown-Grant, Exley ε Naftolin, 1970; Butcher, Collins ε Fugo, 1974).

Although the presence of oestradiol in plasma at sacrifice could be taken as a positive indication of ovarian function, due to the extremely cyclic nature of oestradiol release, it could not be assumed from a single plasma sample with negligible oestradiol that ovarian function was inhibited. It was therefore decided to examine the effects of the two drugs on plasma oestradiol levels in intact rats over a period of eight days. Because of the relatively large volume of plasma required for oestradiol determination a method of pooling daily samples for individual rats was devised.

Since the method had the shortcoming that samples were taken at only one time in the day, this time was chosen to correspond with peak oestradiol levels in proestrus in the cycling rats (Yoshinaga, Hawkins & Stocker, 1969; Brown-Grant, Exley & Naftolin, 1970; Butcher, Collins & Fugo, 1974). Therefore the oestradiol concentration in the cumulative sample pooled from eight consecutive daily samples from a control rat is probably the result of the high oestradiol concentrations in the two proestrus samples being diluted by the much lower concentrations of the other daily samples.

The levels of oestradiol in the cumulative plasma samples covering the 5th to the 12th day of perphenazine administration were on the borderline of sensitivity of the radioimmunoassay. This means that, unless plasma oestradiol levels rose rapidly outside the time of sampling, perphenazine treatment profoundly reduced oestradiol secretion. Although it has been known for some time that perphenazine treatment induces a constant di-oestrus-type vaginal smear pattern (Ben-David, 1968), which is indicative of loss of ovarian cyclicity, as far as I am aware no other measurements of the long term effect of perphenazine on plasma oestradiol have been reported. Chatterton, Chien and Ward (1974) reported reduced levels of serum oestradiol after three daily doses of perphenazine, but in such a time the full effect of inhibiting the oestrous cycle will not have been observed. Also, in the female rat extremely low levels of oestradiol were also found up until the 10th day of pseudo-

pregnancy, a condition which is also associated with a constant dioestrus-type smear (Welschen, Osman, Dullart, de Greef, Uilenbroek ε de Jong, 1975).

The reduction in oestradiol secretion by the ovary probably results from the inhibition of the preovulatory release of luteinising hormone, due partly to a direct action of prolactin (Beck, Engelbart, Gelato ε Wuttke, 1977), and also to the raised levels of plasma progesterone (Chatterton, Chien ε Ward, 1974; Chatterton, Chien, Ward ε Miller, 1975; Wynn, Harris ε Chatterton, 1976). Since prolactin is luteotrophic in the rat (Astwood, 1941; Evans, Simpson, Lyons ε Turpeinen, 1941; Everett, 1956), and high prolactin levels are associated with increased progesterone secretion and a constant dioestrus-type vaginal smear (Chatterton, Chien ε Ward, 1974; Welschen, Osman, Dullart, de Greef, Uilenbroek ε de Jong, 1975; Tomogane, Ota ε Yokoyama, 1975), it is possible that oestradiol levels are suppressed not only by perphenazine-induced hyperprolactinaemia, but by all other procedures which elevate plasma prolactin sufficiently to arrest the vaginal smear pattern in dioestrus.

Oestradiol levels were also extremely low in plasma collected at sacrifice in the non-tumour-bearing rats given perphenazine. However three of the rats in the group of intact tumour-bearing given perphenazine had sacrifice oestradiol levels at least twice as high as the limit of sensitivity of the assay. The difference in oestradiol levels between the two perphenazine-treated groups was probably due to the fact that all non-tumour-

bearing rats used for the cumulative collection study were sacrificed on the 12th day of treatment whereas treatment was sometimes a few days longer in the tumour-bearing rats. It is possible that with more than 12 days of treatment some of the tumour-bearing rats were about to start cycling again.

The levels of oestradiol in both cumulative and sacrifice plasma samples from non-tumour-bearing rats given CB 154 did not differ significantly from those of the control group. In the study on tumour-bearing rats, however, the plasma levels of oestradiol in CB 154-treated rats at sacrifice were higher than those of the corresponding control group. It is possible that this was a chance observation, but all animals were in dioestrus at sacrifice. The observation may merit further investigation since it has been observed that ovaries are considerably enlarged by CB 154 treatment (Heuson, Waelbroeck-van Gaver ε Legros, 1970; Gala ε Boss, 1975). The difference in oestradiol levels, if real, may indicate a difference in overall output, or shift in the timing of the oestradiol surge.

Earlier reports have stressed the heterogeneous nature of rat mammary tumours induced by DMBA with regard to their histological appearance (Huggins, Briziarelli ε Sutton, 1959; Young ε Hallowes, 1973), biochemical properties (Hilf, Goldenberg, Gruenstein, Meranze ε Shimkin, 1970), spontaneous growth rate (Young, Cowan ε Sutherland, 1963; Heise ε Gorlich, 1966), and response in growth rate to various hormonal manipulations (Segaloff, 1968;

Nagasawa, Chen ε Meites, 1973; Bradley, Kledzik ε Meites, 1976). Only tumours which were actively growing at the time of allocation to a treatment group were included for the study of the effect of various treatment regimes on tumour growth and testosterone metabolism. However, it was not possible to determine how the growth of these tumours would have progressed over the same period had the animals remained untreated.

Even after the preselection of actively growing tumours, noticeable variations were observed in the response of these tumours to certain treatment regimes. These variations were related to individuality of the tumours rather than to the differences in the response of individual rats to hormonal manipulation. Despite individual tumour variations, significant differences were observed between the tumour growth patterns of the various treatment groups.

The increased growth rate of DMBA-induced mammary tumours in perphenazine-treated intact rats was similar to that observed on the administration of other drugs capable of raising plasma prolactin levels (Meites, 1970; Meites, Lu, Wuttke, Welsch, Nagasawa ε Quadri, 1972; Quadri, Kledzik ε Meites, 1973). When perphenazine administration was started after DMBA treatment, but before the initial appearance of tumours, both the number and growth rate of resultant tumours were greater than in control rats (Pearson, Llerena, Llerena, Molina ε Butler, 1969).

The administration of CB 154 to intact rats has been reported to cause an inhibition in the growth of 67% of DMBA-induced mammary tumours (Heuson, Waelbroeck-van Gaver ε Legros, 1970), or to have only a slight inhibitory effect on tumour growth (Cassell, Meites ε Welsch, 1971; Teller, Stock, Hellman, Mountain, Bowie, Rosenberg, Boyar ε Badinger, 1977). Although comparisons are difficult because of the different dosage regimes adopted (which may account for the discrepancy in the published results), the general inhibition in tumour growth without much reduction in mean tumour size found in this study appears to lie between the differing effects described by the other workers.

The alterations in tumour growth rate observed after the administration of perphenazine or CB 154 to intact rats are in accord with the accepted view that prolactin plays an important role in maintaining and stimulating the growth of DMBA-induced mammary tumours. However tumour growth rate was not always stimulated by perphenazine nor did all tumours regress on CB 154 administration. Others have found that some DMBA-induced mammary tumours are prolactin insensitive or independent (Bradley, Kledzik ε Meites, 1976), an observation which may be related to the presence of prolactin receptors in some, but not all, tumours (de Sombre, Kledzik, Marshall ε Meites, 1976). Kelly, Bradley, Shiu, Meites ε Friesen (1974) reported a continuous range of values for prolactin receptor content in DMBA-induced mammary tumours which, they claimed, correlated directly with prolactin responsiveness.

In the present study all tumours regressed in ovariectomised rats given only corn oil vehicle. In contrast, other workers have generally found that only 75-90% of DMBA-induced mammary tumours regress after ovariectomy (Young, Cowan ε Sutherland, 1963; Teller, Kaufman, Bowie ε Stock, 1969; Bradley, Kledzik ε Meites, 1976). Although the number of tumours described in the present study was small, ovarian independent tumours have very rarely been found in this colony of rats (Scott, Murphy ε Hawkins, 1979). The reason for this high ovarian dependency may in part be due to the early age at which the operation was performed (usually less than 120 days after DMBA administration). It has been shown that the response to ovariectomy diminishes with increasing age (Griswold ε Green, 1970; Bradley, Kledzik ε Meites, 1976).

Since in the female rat prolactin secretion is maintained by oestradiol and therefore diminishes on ovariectomy, there has been much debate concerning the direct role of oestradiol in maintaining mammary tumour growth. In the present study, the elevation of plasma prolactin by perphenazine delayed, but in general did not prevent, the regression of tumours in ovariectomised rats. Similar tumour growth patterns have also been observed in ovariectomised-plus-adrenalectomised rats whose prolactin levels were raised by median eminence lesions (Welsch, Clemens ε Meites, 1969; Sinha, Cooper ε Dao, 1973), or administration of ovine prolactin (Nagasawa ε Yanai, 1970). These findings suggest that eventually, ovarian hormones are required for the maintenance of

tumour growth by prolactin. The greater regression of tumours in ovariectomised rats given corn oil compared to intact rats given CB 154, despite both groups having low prolactin levels, is also indicative of ovarian hormones playing a role in mammary tumour growth other than by maintaining the secretion of pituitary prolactin.

On the other hand, since some tumours in ovariectomised rats given perphenazine were still growing at the end of the treatment period it is difficult to dispute the claims that prolactin in the absence of ovarian hormones can maintain tumour growth (Kim & Furth, 1960; Pearson, Llerena, Llerena, Molina & Butler, 1969). Because the tumours were required for in vitro studies at the end of the treatment period, it was not possible to ascertain whether the tumours in the group of ovariectomised rats given perphenazine would have regressed after ovariectomy alone or would have regressed spontaneously in the same period without treatment. These two possibilities could have been checked respectively, by discontinuing perphenazine treatment or by discontinuing treatment and grafting in ovaries. Since the present results and those of others (Leung, Sasaki & Leung, 1975) have demonstrated marked differences in the requirements of individual tumours for ovarian hormones, some of the discordant results which have been presented regarding the requirement for ovarian hormones may be due to tumour selection.

It is generally assumed that oestradiol is the ovarian hormone required for tumour growth since ovari-

ectomy-induced tumour regression is reversed by its administration (Sterental, Dominguez, Weissman ε Pearson, 1963; Dao, 1964; Bradley, Kledzik ε Meites, 1976). However in the present study it was found that, in intact rats, perphenazine could reduce plasma oestradiol levels to around the limits of sensitivity of the radioimmunoassay and yet stimulate tumour growth. As already discussed it would be intriguing to know if all methods used to achieve prolonged hyperprolactinaemia also block oestradiol secretion. This possibility appears to have been overlooked in most publications regarding the effect of raised plasma prolactin levels on mammary tumour growth and indeed it has been suggested, without evidence, that prolactin may enhance oestradiol secretion (Cole, 1974; Welsch ε Nagasawa, 1977). In view of the fact, that in both intact and ovariectomised rats given perphenazine, plasma prolactin levels were raised equally, and plasma oestradiol levels were barely detectable, it is necessary to examine the possible reasons for the marked difference in tumour growth seen in these two groups.

One possibility is that oestradiol present in the plasma of intact rats given perphenazine, although reduced to a level which was difficult to estimate, was sufficient to permit a stimulatory action of prolactin on tumour growth. A daily dose of oestradiol as low as 0.01 μg was sufficient to stimulate tumour growth after DMBA administration (Huggins, Grand ε Brillantes, 1961), or to stimulate the growth of regressed tumours in ovari-

ectomised-plus-adrenalectomised rats receiving prolactin (Leung, Sasaki ε Leung, 1975). This quantity is far below the daily dose of 3.75 µg considered as appropriate replacement therapy in ovariectomised rats (Barnes ε Eltherington, 1973).

An alternative possibility is that the other major ovarian hormone, progesterone, plays a role in tumour growth and may account for the difference between the two groups. Although not measured in the present study, it has been reported that the progesterone levels in the blood of intact female rats 24 hours after the last of 5 or 6 daily doses of perphenazine (5 mg/kg body weight) were significantly elevated compared to those of control rats (Chatterton, Chien, Ward ε Miller, 1975; Wynn, Harris ε Chatterton, 1976). Treatment started in oestrus resulted in an 18-fold increase in plasma progesterone levels compared to a 4-fold increase when started in proestrus. The authors concluded, that the raised progesterone levels were due to an increased secretion of the steroid from the corpus luteum brought about by the luteotrophic action of prolactin (Astwood, 1941; Evans, Simpson, Lyons ε Turpeinen, 1941; Everett, 1956). From these results it is probable that progesterone levels of intact rats in the present study were also elevated by perphenazine for at least the first half of the treatment period.

After ovariectomy progesterone levels fall to about one tenth of those in the intact rat (Feder, Resko ε Goy, 1968; Resko, 1969; Piva, Gagliano, Motta ε Martini, 1973).

The residual levels result from the adrenal production of progesterone (Holzbauer, Newport, Birmingham ε Traihou, 1969; Mann ε Barraclough, 1973; Shaikh ε Shaikh, 1975; Brown, Courtney ε Marotta, 1976; Campbell, Schwartz ε Firlit, 1977), which can be stimulated by ACTH and prolactin (Feder ε Ruf, 1969; Resko, 1969; Piva, Gagliano, Motta ε Martini. 1973). However, the degree by which progesterone levels can be stimulated in the ovariectomised rat appears to be considerably less than in the intact rat. From the evidence available it is probable that during perphenazine treatment progesterone levels were considerably higher in intact than in ovariectomised rats.

Although the role of progesterone in the growth of established DMBA-induced tumours has received little attention there is some evidence that it may have a stimulatory effect. DMBA-induced mammary tumours contain specific cytoplasmic receptors for progesterone (Terenius, 1973; Asselin, Labrie, Kelly, Philibert ε Raynaud, 1977), the levels of which appear to be under the control of oestradiol and prolactin (Kelly, Asselin, Labrie ε Raynaud, 1977), and are greatly reduced by combined ovariectomy-plus-adrenalectomy or hypophysectomy (Asselin, Melançon, Moachon ε Bélanger, 1980). Progesterone has been reported to increase DNA synthesis in organ culture of DMBA-induced tumours (Pasteels, Heuson, Heuson-Stiennon ε Legros, 1976). Treatment of rats with progesterone after DMBA administration has been shown to stimulate subsequent mammary tumour development (Huggins,

Moon ε Morii, 1962; Jabara ε Harcourt, 1970; Kelly, Asselin, Labrie ε Raynaud, 1977). It has been proposed, that progesterone exerts its stimulatory effect at tumour level (Jabara, Marks, Summers ε Anderson, 1979). It has also been proposed that progesterone may play an important role in regulating the growth of DMBA-induced mammary tumours, during pseudopregnancy, pregnancy and the postpartum period (Dao, 1964; McCormick ε Moon, 1965 ε 1967; Leung, 1977).

Recent reports suggest that progesterone is essential in maintaining the growth of the transplantable, hormone dependent MTW-9 mammary tumour in rats. The inhibition of the growth of this tumour by prostaglandin F2α has been attributed to a fall in progesterone levels since progesterone administration restores tumour growth (Jubiz, Frailey ε Smith, 1979). Progesterone also prevents ovariectomy-induced regression of MTW-9 tumours grown in rats with elevated prolactin levels induced by perphenazine (Diamond, Koprak ε Hollander, 1980).

The fact that progesterone secretion is enhanced by perphenazine in intact rats and the likelihood that progesterone has a stimulatory effect on mammary tumour growth raises the possibility that progesterone could support the stimulatory effect of prolactin at tumour level when oestradiol secretion is reduced in intact rats given perphenazine. When progesterone was given to ovariectomised rats tumour regression was not prevented (Kelly, Asselin, Labrie ε Raynaud, 1977), but since progesterone does not share the capacity of oestradiol to

stimulate prolactin secretion (Chen ε Meites, 1970), it would be necessary to repeat this study in rats whose prolactin levels were maintained artificially.

Although it is widely accepted that prolactin is essential for the growth of the majority of DMBA-induced mammary tumours in the rat and that an increase in plasma prolactin levels stimulates tumour growth by a direct action at tumour level, from the information now available two highly speculative propositions may be made concerning an indirect stimulatory effect of prolactin on tumour growth in intact rats. It was suggested above that, in place of oestradiol, progesterone may act synergistically with prolactin but it is possible that the raised plasma progesterone levels in hyperprolactinaemic rats could have a direct stimulatory effect at tumour level and account in part for the increase in tumour growth rate attributed to prolactin. In addition, it is possible that if the raised plasma levels of oestradiol at proestrus in the cycling rat are similar to those achieved when tumour growth is inhibited by high doses of oestradiol (Huggins ε Yang, 1962; Meites, Cassell ε Clark, 1971), then abolition of the proestrus surge of oestradiol in hyperprolactinaemic animals would reduce this cyclic inhibitory effect of oestradiol, and thus permit faster tumour growth.

Statistical analysis of the metabolism of testosterone and its conversion to 5α-dihydrotestosterone and 5α-androstanediol by the tumours from the five treatment groups was performed using variance analysis plus

Dunnett's test or Student-Newman-Keuls test. The Wilcoxon rank test, which was used to compare results from control and perphenazine-treated groups in two publications (Buchan, Fraser ε Miller, 1976; Buchan ε Miller, 1978), was inappropriate for the larger number of intercomparisons made between the five groups.

To compare the effects of different treatment regimes on a given parameter it is preferable that all groups should be alike in every respect at the start of treatment. However, on allocation, significant inter-group differences occurred in two parameters. It was found that, purely by chance, the mean time between carcinogen administration and allocation to a treatment group was greater in the group of intact rats given perphenazine than in the intact control group and that means of initial tumour sizes of both intact rats given CB 154 and the ovariectomised control group were greater than those of the other groups. This second difference was deliberately made in order to obtain sufficient tumour mass after treatments which were expected to cause tumour regression. Although it is less than ideal to have differences between groups before starting treatment, neither the time between DMBA and start of treatment nor the size or growth rate of tumours showed any correlation with the metabolism of testosterone by tumours in any of the five groups. It is therefore assumed that the differences in the metabolism of testosterone by tumours from the various groups were due to the specific treatments and not to these initial differences in age and tumour size.

The basic observation made was that the in vitro metabolism of testosterone by established tumours from intact rats given perphenazine for two weeks was higher than that of tumours from intact control rats. 5α-Reduction, which accounted for most of the testosterone metabolism, was also significantly higher in tumours from the perphenazine-treated rats, as was the net formation of the individual metabolites, 5α-dihydrotestosterone and 5α-androstanediol. These results extend the earlier work of Miller (1976 a), which demonstrated that tumours from a group of rats given perphenazine continuously from 30 days of age exhibited higher 5α-reduction of testosterone than those of a control group. From that study it was not possible to determine whether the treatment acted at the stage of tumour induction to promote the development of a tumour with different properties, or whether it exerted an effect upon the growing tumour. The results of the present study show that it is possible to stimulate 5α-reductase activity in established tumours.

Since the present investigation of plasma oestradiol levels and reports in the literature have revealed that perphenazine treatment cannot raise plasma prolactin levels without altering the levels of other hormones the increase in 5α-reduction of testosterone by tumours from perphenazine-treated rats may have been caused by one or more different factors. Testosterone metabolism was therefore measured in tumours from rats which had undergone other hormonal manipulations in an

attempt to determine which factors may be involved in the control of tumour 5α-reductase activity.

The possibility that perphenazine itself may act directly at tumour level to increase 5α-reductase activity was not supported by the results from in vitro studies in which there was no evidence of a stimulation of 5α-reductase activity in tumours incubated in the presence of perphenazine. However, this type of study is insufficient to discount a direct action of perphenazine on the tumour in vivo and despite the fact that perphenazine is always assumed to act on the mammary gland and mammary tumours via an effect on hormonal secretion, it is very difficult to eliminate the possibility of the drug working directly. It would be informative to assess the effect of elevating plasma prolactin by some other means, preferably without the use of drugs, on tumour 5α-reductase activity.

The observation that in both intact and ovariectomised rats the perphenazine-induced elevation of plasma prolactin levels was accompanied by an increase in tumour 5α-reductase activity compared to the respective controls suggests that the two parameters may be positively related. The diminished levels of plasma prolactin and lower levels of testosterone metabolism by tumours in ovariectomised controls compared to intact controls is in general agreement with such a relationship. However prolactin levels were similarly reduced by the administration of CB 154 to intact rats, but testosterone metabolism parameters did not differ from

those of the control group. Moreover, although perphenazine elevated plasma prolactin levels to the same extent in ovariectomised and intact rats the level of tumour 5α-reduction was significantly lower in the former group. On the basis of these observations the differences between tumours of the five treatment groups in their ability to metabolise testosterone cannot be accounted for solely by differences in plasma prolactin levels. It is nevertheless possible to propose a purely stimulatory role for prolactin in tumour 5α-reductase activity which is dependent on the growth of new tumour tissue during the treatment period.

In other target tissues prolactin appears to have a mainly inhibitory effect. The formation of 5α-dihydrotestosterone from testosterone by canine prostate was inhibited in dogs given prolactin and stimulated in those given CB 154 (Helmerich ε Altwein, 1976). However the total turnover of testosterone by the prostate was not inhibited by prolactin administration. The administration of CB 154 to humans also increased the relative formation of 5α-dihydrotestosterone from testosterone by carcinoma of the prostate but a significant reduction in androstanediol formation was also observed (Kurth, Jacobi, Sinterhauf ε Altwein, 1977). It has been shown, that prolactin has an inhibitory effect on the 5α-reduction of 4-androstenedione and corticosterone by the adrenal cortex (Witorsch ε Kitay, 1970 ε 1972; Gustafsson ε Stenberg, 1975; Witorsch ε Edwards, 1976). Inhibition of adrenal 5α-reduction of testosterone and

4-androstenedione has been reported following prolactin administration to male, female and pseudohermaphrodite rats (Goldman ε Shapiro, 1977). In pregnant rats, 5α-reduction of progesterone by the ovary increased when ergocornine was administered alone, but not when accompanied by prolactin administration (Zmigrod, Lindner ε Lamprecht, 1972). Hypophysectomy decreases, and subsequent prolactin administration increases, 5α-reduction of testosterone by rat liver (Schriefers, Keck, Klein ε Schroder, 1975).

When the report that oestradiol administration to ovariectomised rats reduced the capacity of DMBA-induced tumours to convert testosterone to 5α-dihydrotestosterone and 5α-androstanediol (Miller, 1976 c) and the present observation that plasma oestradiol levels in intact rats were drastically lowered by perphenazine administration are considered together, it is possible to postulate, that the increased 5α-reduction of testosterone by DMBA-induced tumours from intact rats given perphenazine was due to the removal of the inhibitory effect of oestradiol. However other intergroup differences cannot be attributed to inhibitory effects of oestradiol. Although tumour 5α-reductase activity was greater in ovariectomised rats given perphenazine than in ovariectomised control rats, there was no difference between the extremely low levels of oestradiol in both groups. The 5α-reduction of testosterone by tumours of the intact, CB 154-treated rats was greater than that of the ovariectomised control group which had, in fact, lower plasma

oestradiol levels. Moreover, in the present study, tumours from the ovariectomised control group metabolised significantly less testosterone than tumours from the intact control group. However, the formation of 5α-reduced products was only numerically and not significantly lower in the ovariectomised group. The results from ovariectomised and intact control groups in the present study are at variance with the results of Miller (1976 c), who found that ovariectomy caused a numerical increase in tumour 5α-reduction and a significant increase in the formation of 5α-androstanediol. The differences between the two sets of results lie mainly with the relative amounts of 5α-androstanediol formed by tumours from the control groups. The two hour incubation period of the earlier study as opposed to the one hour incubation period used in the present study may have contributed to this difference. So also may the fact that all intact control rats in the present study were in dioestrus at sacrifice whereas those of the earlier study were not killed in any specific stage of the cycle.

Alterations in the levels of circulating oestrogens have been reported to affect 5α-reductase activity in other tissues. However it is sometimes difficult to determine whether the effects observed are due to the direct action of oestrogens or due to secondary changes in the levels of other hormones such as prolactin. The administration of oestrogens to humans, dogs or rats has been shown to result in a general inhibition of the net

formation of 5α-dihydrotestosterone from testosterone by prostatic tissues (Farnsworth, 1970; Griffiths, Harper, Groom, Pike, Fahmy ε Pierrepoint, 1970; Belham ε Neal, 1971; Leav, Morfin, Ofner, Cavazos ε Leeds, 1971; Danutra, Harper ε Griffiths, 1973; Fencl ε Villee, 1973; Jenkins ε McCaffery, 1974; Yamanaka, Shimazaki, Imai, Sugiyama ε Shida, 1975). Castration was found to stimulate and oestradiol administration to inhibit 5α-reductase activity in the rat adrenal whether measured using 11-substituted, C-21 precursors (Kitay, Coyne ε Swygert, 1970), or C-19 precursors (Maynard ε Cameron, 1973). In male rats, oestrogen administration has been reported to stimulate hepatic 5α-reductase activity (Schriefers, 1967; Dieringer, Lamartiniere ε Lucier, 1979), but the higher 5α-reductase activity in the liver of female rats was only slightly lowered by ovariectomy, and unaffected by the administration of oestrogens (Dieringer, Lamartiniere ε Lucier, 1979). Pituitary hormones appear to be more important than sex steroids in the control of hepatic 5α-reductase activity.

Plasma progesterone levels were not measured during these studies since attention was directed towards the two main hormones thought to be important for the growth of DMBA-induced mammary tumours, prolactin and oestradiol. The probable effects of perphenazine on progesterone levels in intact and ovariectomised rats have already been discussed in connection with tumour growth, but are also of interest in connection with the results of testosterone metabolism by tumours.

The increase in plasma progesterone levels induced by perphenazine in intact rats (Chatterton, Chien, Ward ε Miller, 1975; Wynn, Harris ε Chatterton, 1976), and the decrease following ovariectomy (Feder, Resko ε Goy, 1968; Resko, 1969; Piva, Gagliano, Motta ε Martini, 1973) correspond with an increase and a decrease respectively in the in vitro metabolism of testosterone by tumour similarly treated groups in this study. It is probable that perphenazine increased plasma progesterone levels in ovariectomised rats, as these animals have raised plasma prolactin levels, which in turn stimulate the release of progesterone from the adrenal (Piva, Gagliano, Motta ε Martini, 1973). Since most of the progesterone released during perphenazine treatment of intact rats appears to be of luteal origin (Chatterton, Chien, Ward ε Miller, 1974), and prolactin only caused a threefold increase in the low levels of progesterone in the ovariectomised rats (Piva, Gagliano, Motta ε Martini, 1973), it is unlikely that the progesterone levels in ovariectomised rats given perphenazine were elevated to the same extent as those in intact rats. If this were the case then progesterone levels would again correspond well with the 5α-reductase activity in tumours from ovariectomised rats given perphenazine, which was higher than that of tumours from the ovariectomised control group but not as high as that in tumours from intact rats given perphenazine.

The administration of CB 154 in doses similar to those used in the present study was found to reduce

plasma prolactin levels throughout the cycle and prevent pregnancy despite repeated mating (Wuttke ε Döhler, 1973; Döhler ε Wuttke, 1974). In these rats the peak levels of progesterone which occur in proestrus were suppressed but the levels at other points in the oestrous cycle remained fairly constant, at or slightly above those of untreated cycling rats. The apparent ability of CB 154 to reduce the cyclic variation of plasma progesterone levels without causing much of a change in overall output may be pertinent to the present discussion since mean tumour 5α-reductase activity was similar in control and CB 154-treated rats but the variation in 5α-reductase levels was markedly less in tumours of the CB 154-treated group.

Therefore, on the basis of these other studies, there is a strong possibility that the effect of the 5 treatment regimes on plasma progesterone levels would have paralleled the changes observed in the 5α-reduction of testosterone by the tumours in these groups. It would have been interesting not only to have compared progesterone levels with 5α-reductase activity on a group basis, but perhaps also on an individual basis. An investigation directed specifically towards examining the effects of progesterone on both the growth and 5α-reductase activity of DMBA-induced tumours is perhaps warranted.

In the male rat the administration of progestagens has been shown to stimulate 5α-reductase in the liver, but to be without effect on 5α-reductase of the prostate

(Altman, Gordon, Southren, Vittek ε Wilker, 1972; Albin, Vittek, Gordon, Altman, Olivo ε Southren, 1973; Moore ε Wilson, 1973).

Testosterone has also been shown to enhance its own metabolism in that castration lowers and testosterone administration increases testosterone 5α-reductase activity in rat prostate (Shimazaki, Ohki, Matsuoka, Tanaka ε Shida, 1972; Moore ε Wilson, 1973; Yamanaka, Kirdani, Saroff, Murphy ε Sandberg, 1975), and in the coagulating gland and seminal vesicles of the mouse (Alison ε Wright, 1979).

Peripheral plasma levels of testosterone in the female rat vary with the oestrous cycle from about 80-90 pg/ml in oestrus and metoestrus to about 170-240 pg/ml in proestrus (Dupon ε Kim, 1973; Rosenfeld, Jones, Dupon, Fang ε Anderson, 1975; Terranova, Saidapur ε Greenwald, 1980). Plasma testosterone levels were unaltered by adrenalectomy but fell to non-detectable levels after ovariectomy (Frankel, Mock, Wright ε Kamel, 1975). In agreement with these observations, Terranova, Saidapur and Greenwald (1980) have shown that the ovary, and in particular corpora lutea, are important in the production of both testosterone and its potential precursor, 4-androstenedione. However, it has also been shown that the adrenal gland of the female rat is capable of converting progesterone to testosterone in vitro (Vinson, Bell ε Whitehouse, 1976). It has been suggested that the elevated plasma testosterone levels found in pregnant rats were due to a stimulatory effect of prolactin (Weizenbaum, Adler ε Ganjam, 1979). On the basis

of these results it is possible that perphenazine, by increasing prolactin secretion, may have elevated plasma testosterone in intact rats but it is difficult to speculate over testosterone levels in ovariectomised rats given perphenazine and intact rats given CB 154. However it is questionable whether such alterations in testosterone levels, if in fact true, could have affected 5α-reductase activity since the levels of testosterone in female rats are relatively low.

As indicated by the constant dioestrus-type smear and other morphological characteristics perphenazine treatment appears to inhibit the secretion of gonadotrophins in the female rat (Verlardo, 1958; Ben-David, 1968). Whilst it has been demonstrated that these hormones can affect 5α-reductase activity in their classical target organs (Fölman, Sowell ε Eik-Nes, 1972; Terakawa, Kondo, Aono, Kurachi ε Matsumoto,1978), evidence of gonadotrophins having a direct effect on DMBA-induced rat mammary tumours appears to be lacking. It has been reported that perphenazine does not affect the secretion of growth hormone (MacLeod, Fontham ε Lehmeyer,1970).

Although some evidence for the stimulation of ACTH release by phenothiazines has been presented (Smith, Maickel ε Brodie, 1963), and perphenazine has been reported to elevate corticosterone levels (Chatterton, Chien ε Ward, 1974), over short periods of administration, others have found no difference in adrenal weight or plasma corticosterone levels after more prolonged administration of either perphenazine or CB 154 (Cameron ε

Scarisbrick, 1973). More recently Vasquez and Kitay (1978) have shown that although prolactin augments stress-induced changes in plasma corticosterone levels it has no effect on resting levels.

It is also necessary to consider the possibility, that the measurement of 5α-reductase activity was affected by the presence of hormones in the tumour after excision. The concentrations of hormone present in the DMBA-induced mammary tumours after their removal were not measured in the present study and there appears to be no relevant information available regarding the possible effect of these treatment regimes on hormone levels in tumours.

It is possible that hormones present in the tumour could have a direct effect on the 5α-reductase enzyme or affect NADPH availability by altering the activity of endogenous and added glucose-6-phosphate dehydrogenase. Several steroids have been shown to reduce glucose-6-phosphate dehydrogenase activity in vitro (Raineri ε Levy, 1970; Benes ε Oertel, 1970).

The in vitro addition of prolactin has been reported to inhibit 5α-reductase activity in DMBA-induced rat mammary tumours (Miller, 1976 d), and in rat and guinea-pig prostate (Mawhinney, Belis ε Lloyd, 1975; Manandhar ε Thomas, 1976). There is evidence that in several tissues both progesterone and testosterone serve as substrates for 5α-reductase and, in common with other 4-ene,3-ketosteroids which are unsubstituted at the 11-position, competitively inhibit 5α-reductase

(Voigt, Fernandez ε Hsia, 1970; Frederiksen ε Wilson, 1971; Hsia, 1971; Voigt ε Hsia, 1973; Mauvais-Jarvis, Kuttenn ε Wright, 1975; Patwardhan ε Lanthier, 1975; Smethurst ε Williams, 1975; Saksena, Lau ε Chang, 1976; Eckstein ε Nimrod, 1977; Krauze ε Karavolas, 1978). It has recently been proposed, on the basis of the close similarity between the 5α-reduction of testosterone and progesterone, that both are metabolised by the same enzyme in DMBA-induced rat mammary tumours.(Mori, Tominaga ε Tamaoki, 1978; Tamaoki, Mori, Kitamura ε Tominaga, 1978). However, if the concentrations of hormones in DMBA-induced mammary tumours are directly related to their plasma levels, it seems unlikely that the presence of these hormones in the excised tumours could have accounted for the differences in tumour 5α-reductase activity between the groups, since the increase in 5α-reductase activity of tumours from perphenazine-treated animals was in fact associated with raised plasma levels of prolactin and probably also progesterone and testosterone.

An inhibitory effect of oestrogens on the 5α-reduction of testosterone by human, dog and rat prostate in vitro has been described by several workers (Farnsworth, 1969; Baulieu ε Robel, 1970; Belham ε Neal, 1971; Groom, Harper, Fahmy, Pierrepoint ε Griffiths, 1971; Jenkins ε McCaffery, 1974; Patwardhan ε Lanthier, 1975; Bard ε Lasnitzki, 1976 ε 1977). Although the concentrations of oestradiol used in these studies were higher than that expected physiologically, Eckstein and Nimrod

(1977) have demonstrated the non-competitive inhibition of 5α-reductase of the rat ovary with a concentration of oestradiol which may be of physiological relevance in the ovary. In the DMBA-induced rat mammary tumour, oestradiol was also found to have an inhibitory effect on 5α-reductase activity in vitro (Miller, 1976 b). Despite the fact that differences between the five treatment groups could not be accounted for by alterations in plasma oestradiol levels, two trends were observed which indicate that plasma oestradiol levels at sacrifice may be inversely related to tumour 5α-reductase levels measured in vitro. All r values for the simple coefficient of correlation between plasma oestradiol at sacrifice and tumour 5α-reduction in the three groups of intact animals and in the cycle study were high and negative suggesting a possible inverse relationship between the two parameters. Furthermore in the intercomparison of groups at different stages of the cycle all but one of the tumours from the rats killed in proestrus (the stage with the high plasma oestradiol concentrations) showed below average 5α-reductase activity. However, the difference between the different stages of the cycle did not attain significance at the 5% level. Although neither of these observations were in themselves statistically significant, the trend in these results is in agreement with the inhibitory effects of oestradiol on 5α-reductase activity of DMBA-induced mammary tumours in vitro (Miller, 1976 b), and also that attributed to oestradiol after its administration in vivo (Miller, 1976 c).

When comparing the results of the intact perphenazine-treated rats with those of the intact control group it could be argued, that the increased metabolism of testosterone by tumours from the former group simply reflects the increased tumour growth rate resulting from hormonal changes. The reduced levels of testosterone metabolism by tumours from ovariectomised control rats would support the argument of tumour growth being related to testosterone metabolism. However tumours of both intact rats given CB 154 and ovariectomised rats given perphenazine, which were on average regressing at the time of sacrifice, showed no difference in their ability to metabolise testosterone when compared with the growing tumours of the intact control group. Moreover within groups there was no obvious correlation between tumour size or growth and any of the parameters of testosterone metabolism. This observation is of particular significance in the group of intact rats given CB 154 and in the group of ovariectomised rats given perphenazine, since in both of these groups tumour growth ranged from rapidly growing to rapidly regressing at the time of sacrifice. King, Panattoni, Gordon and Baker (1965) were also unable to find a relationship between tumour growth and the metabolism of testosterone. The observation, that in fast growing tumours of perphenazine-treated rats the 5α-reduction of testosterone was increased whilst the metabolism of DHA was unaltered (Miller, 1976 a), also provides evidence that the increase in 5α-reductase activity is specific and not just

a reflection of a general increase in metabolic activity. It may, however, be interesting to compare 5α-reduction in growing, static and spontaneously regressing DMBA-induced tumours of otherwise untreated rats.

Although there appears to be no direct correlation between the growth of a tumour and its ability to metabolise testosterone, this does not dismiss the possibility that the growth of new tumour tissue must occur, in order that an increase in 5α-reductase due to another stimulus may be observed. It was shown that the induction of hepatic 5α-reductase by progesterone was due to the synthesis of new enzyme protein (Altman, Gordon, Southren, Vittek ε Wilker, 1972), and that the rise in prostatic 5α-reductase by testosterone coincided with maximal increase in RNA and DNA content.(Moore ε Wilson, 1973).

It has been claimed that in cancer of the prostate, 5α-reductase activity is lower in poorly differentiated tissues (Morfin, Charles ε Floch, 1979). In normal and benign human prostate 5α-reductase activity resided predominantly in stroma with very little in epithelium (Cowan, Cowan ε Grant, 1976; Cowan, Cook, Cowan, Grant, Sirett ε Wallace, 1979). Possible relationships between steroid metabolism and histological features of the DMBA-induced mammary tumours were not investigated in this study, but the lack of correlation between tumour DNA content and parameters of testosterone metabolism suggests that the latter are unrelated to cellularity.

The effects of 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol on the size and numbers of growing, static or regressing DMBA-induced tumours have been measured and compared with those of a control group given only injection vehicle.

No regressing tumours were present in the control group at the pretreatment period, but a significant number spontaneously regressed during the study period. Overall proportions of growing, static and regressing tumours were similar to those reported by Heise and Gorlich (1966), Teller, Stock, Stohr, Merker, Kaufman, Escher and Bowie (1966), but not to those reported by Young, Cowan and Sutherland (1963), who observed a lower percentage of growing tumours.

Both 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol (1 mg daily over a period of approximately 12 days) strongly inhibited tumour growth both in terms of mean tumour size and the proportions of growing, static and regressing tumours. Over 75% of the tumours in these groups underwent regression. In contrast, 5α-androstane-3β,17β-diol, administered in the same manner, did not cause tumour regression.

Such effects of these free steroids on the growth of DMBA-induced mammary tumours have not been previously reported. However, administration of 5α-dihydrotestosterone, in similar doses but given over a longer period, caused regression of transplanted hormone dependent mammary tumours (Huggins ε Mainzer, 1957) and 3-MC-induced rat mammary tumours (Huggins, Briziarelli ε Sutton, 1959).

About 78% of 3-MC-induced tumours regressed, a proportion which is similar to the value found in the present study.

Administration of 5α-androstane-3α,17β-diol as the dipropionate has been reported to inhibit the growth of DMBA-induced rat mammary tumours (Griswold, Skipper, Laster, Wilcox ε Schabel, 1966; Teller, Stock, Stohr, Merker, Kaufman, Escher ε Bowie, 1966), but no information regarding the effect of 5α-androstane-3β,17β-diol on DMBA-induced rat mammary tumours appears to have been published. However in a study of a series of androgens, which did not include 5α-androstanediols, Huggins and Mainzer (1957) found that, compared with 3α-isomers, steroids with 3β-hydroxyl groups were far weaker inhibitors of the growth of a transplanted mammary fibroadenoma.

Several groups have reported that doses of testosterone propionate, similar to those used for its 5α-reduced metabolites in this study, had an inhibitory effect on the growth of DMBA-induced mammary tumours (Griswold, Skipper, Laster, Wilcox ε Schabel, 1966; Heise ε Gorlich, 1966; Teller, Stock, Stohr, Merker, Kaufman, Escher ε Bowie, 1966; Costlow, Buschow ε McGuire, 1976).

In general the cessation of treatment with 5α-dihydrotestosterone or 5α-androstane-3α,17β-diol halted tumour regression, and after a delay growth was observed in some tumours, although not at the rate displayed by the control group. The recovery appeared to be more

restricted in the 5α-androstane-3α,17β-diol-treated rats, but the difference between the two groups was slight. These findings agree with those of Quadri, Kledzik and Meites (1974), who measured tumour size over a three week recovery period after the inhibition of tumour growth by dromostanolone propionate (2α-methyl-17β-hydroxy-5α-androstan-3-one propionate). During the recovery period some resumption of mammary tumour growth was observed but it did not attain that of the control rats. In contrast it had previously been found that complete mammary tumour regrowth had occurred following the termination of treatment with doses of oestradiol benzoate and ergot drugs which had inhibitory effects on tumour growth (Meites, 1972 a ε b). The conclusion reached was that the difference in recovery could be due to differences in the mechanism action of the three agents or to dosage effects.

In addition to tumour size two other parameters were measured in the androgen-treated rats. The oestrous cycle was assessed from daily vaginal smears and plasma prolactin was assayed in blood samples taken before and at the end of treatment and at the end of the recovery period.

5α-Dihydrotestosterone and 5α-androstane-3α,17β-diol, both of which induced extensive tumour regression, also arrested the oestrous cycle as indicated by the persistent dioestrus-pattern of vaginal smears. On average there was a delay of about six days after the cessation of treatment before cycling resumed. The oestrous cycle

appeared to be unaffected by 5α-androstane-3β,17β-diol treatment which did not induce tumour regression.

Plasma prolactin levels in the non-cycling rats treated with 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol were similar to those obtained in cycling control rats in the dioestrus stage of the cycle. However it has been shown that plasma prolactin levels in cycling rats are much lower in dioestrus, than during the oestradiol-induced surge of prolactin in proestrus (Neill, Freeman ε Tillson, 1971; Butcher, Collins ε Fugo, 1974). If, in comparison, the plasma prolactin levels remained constant in the non-cycling rats and the value obtained at the end of treatment was representative of the levels throughout the treatment period, then this result implies that the net output of prolactin was in fact considerably lower in the non-cycling rats of the 5α-dihydrotestosterone- and 5α-androstane-3α,17β-diol-treated groups than in the cycling rats of the control group.

In other situations where constant dioestrus smears are exhibited such as ovariectomy, perphenazine administration (this thesis), and pseudopregnancy (Welschen, Osman, Dullart, de Greef, Uilenbroek, ε de Jong, 1975), plasma oestradiol levels are extremely low. Indeed, the fact that very small amounts of oestradiol are required to break the constant dioestrus pattern in ovariectomised rodents has been used as a highly sensitive and specific bioassay for oestrogens (Allen ε Doisy, 1923; Emmens, 1962). Furthermore androgen treatment has been shown to

induce atrophy of the ovary which was associated with a pattern of vaginal smears whose description resembled that of constant dioestrus (McEuen, Selye ε Collip, 1937; Huggins ε Mainzer, 1957). These effects are probably attributable to the ability of androgens to block gonadotrophin release from the anterior pituitary (Beyer, Jaffe ε Gay, 1972; Swerdloff, Grover, Jacobs ε Bain, 1973; Zanisi, Motta ε Martini, 1973; Döhler, Wong, von zur Mühlen ε Döhler, 1977).

In view of these observations it seems probable that treatment with 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol but not with 5α-androstane-3β,17β-diol resulted in an inhibition of the cyclic release of oestradiol by the ovaries. Since oestradiol is the usual physiological stimulus for prolactin release in the rat (Neill, Freeman ε Tillson, 1971), it is likely that the cyclic release of prolactin was also inhibited. Such a situation would account for plasma prolactin levels in the 5α-dihydrotestosterone- and 5α-androstane-diol-treated rats similar to the low levels found in the dioestrus stage of normal cycling rats. As has already been discussed, both prolactin and oestradiol appear to be important in maintaining the growth of DMBA-induced mammary tumours. The possibility, therefore, arises that a reduction in the levels of these hormones may play a role in tumour regression induced by 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol. The finding that 5α-androstane-3β,17β-diol did not apparently inhibit the hormonal changes of the oestrous cycle and did

not induce significant tumour regression is in keeping with this hypothesis.

Although the inhibitory effects of androgens on the growth of DMBA-induced mammary tumours in the rat may be accounted for simply on the basis of alterations in the circulating levels of hormones, a direct effect at tumour level cannot be dismissed. In fact most results appear to have been interpreted on the basis of androgens acting directly on the tumour. From the observation that concommitant prolactin administration completely abolished the inhibitory effects of dromostanolone propionate on the growth of DMBA-induced mammary tumours, it was concluded that the androgen acted, by blocking the stimulatory effects of prolactin at tumour level (Quadri, Kledzik ε Meites, 1974). This conclusion stimulated a study of the effect of androgen-induced tumour regression on prolactin receptor levels, in which it was found that, although the quantity of prolactin receptors was reduced in most tumours by androgen treatment, the reduction was insufficient to account for the androgen-induced tumour regression (Costlow, Buschow ε McGuire, 1976).

That androgen-induced tumour regression may be mediated via a reduction in plasma levels of prolactin appears to have been overlooked by these workers, on the basis of observations that in male rats testosterone could produce small increments in prolactin secretion in vivo (Meites ε Clemens, 1972; Kalra, Fawcett, Krulich ε McCann, 1973; Shin, Akin, Roberts ε Howitt, 1974).

This effect has been attributed to the conversion of testosterone to oestradiol, which is a more potent stimulator of prolactin secretion (Herbert, Cisneros ε Rennels, 1977; Nolin, Campbell, Nansel ε Bogdanove, 1977; Döhler, Wong ε von zur Mühlen, 1978). If this were the case, then the results obtained with testosterone could not be extrapolated to androgens, such as the 5α-reduced C-19 steroids, which are incapable of forming oestradiol. Moreover in the castrated female rat no elevation of plasma prolactin was detected after the administration of a range of doses of testosterone and 5α-dihydrotestosterone (Döhler, Wong ε von zur Mühlen, 1978). It seems likely that, in female rats, an inhibition of the ovarian release of oestradiol would have an overriding effect and cause an overall decrease in prolactin secretion. It is, therefore, possible that prolactin reversed the androgen-induced tumour regression (Quadri, Kledzik ε Meites, 1974), by restoring plasma prolactin levels, which had been lowered as a result of a decrease in oestradiol secretion. It is possible also that the diminished prolactin receptor levels in tumours of androgen-treated rats reported by Costlow, Buschow and McGuire (1976) were in part due to decreased plasma levels of oestradiol and prolactin. Both of these hormones have been associated with a stimulation in the levels of prolactin receptor in other tissues (Posner, Kelly ε Friesen, 1974 ε 1975; Gelato, Marshall, Boudreau, Bruni, Campbell ε Meites, 1975; Costlow, Buschow ε McGuire, 1975; Kelly, Posner ε Friesen, 1976; Hammond ε Krall, 1979).

In so far as receptor sites which specifically bind 5α-dihydrotestosterone have recently been characterised in the cytosol from DMBA-induced tumours (Ip, Milholland, Kim ε Rosen, 1978; Asselin, Melançon, Moachon ε Bélanger, 1980), it seems probable that androgens can act directly at tumour level. These receptors have a high affinity and low capacity for binding 5α-dihydrotestosterone and resemble those identified in classical androgen target tissues. In common with other tissues which possess specific receptors for 5α-dihydrotestosterone the DMBA-induced mammary tumour also has a large capacity for the conversion of testosterone to 5α-dihydrotestosterone (Gloyna ε Wilson, 1970). However in these tissues the interaction between 5α-dihydrotestosterone and its specific receptors leads to a stimulation of growth. It remains to be seen whether the specific 5α-dihydrotestosterone receptors found in DMBA-induced rat mammary tumours mediate the inhibitory effects of pharmacological doses of androgen on tumour growth.

It may be more relevant that androgens have been shown to bind to oestrogen receptors which are present in substantial concentrations in most DMBA-induced tumours (Powell-Jones, Davies, Wilson ε Griffiths, 1976; Garcia ε Rochefort, 1978). Androgens can induce nuclear translocation of the oestrogen receptor complex and the initiation of certain post-transcriptional events characteristic of receptor binding with oestradiol or synthetic anti-oestrogens (Garcia ε Rochefort, 1978; Nicholson, Davies ε Griffiths, 1978). Although androgens exhibit a

lower affinity for oestrogen receptors than does oestradiol itself it must be taken into account that androgens are given in milligram quantities to ellicit tumour responses whereas only microgram quantities of oestradiol are required to reverse ovariectomy-induced tumour regression. The relationship between the competitive binding of androgens to oestradiol receptors and their antagonistic and possibly agonistic properties, relative to oestrogen action, however requires further elucidation (Rochefort, Capony ε Garcia, 1979).

It is interesting to note that of the steroids tested 5α-androstane-3β,17β-diol, in common with another 3β,17β-hydroxylated steroid, androst-5-ene-3β,17β-diol, showed a much greater affinity towards the oestrogen receptor than other C-19 steroids (Davies, Powell-Jones, Nicholson ε Griffiths, 1977; Rochefort, Capony ε Garcia, 1979). The present finding that, in comparison with the other two 5α-reduced steroids, 5α-androstane-3β,17β-diol was ineffective in causing tumour regression does not lend support to the theory that androgens inhibit the growth of DMBA-induced rat mammary tumours by directly binding to oestrogen receptors (Ip, Milholland, Kim ε Rosen, 1978; Nicholson, Davies ε Griffiths, 1978). This observation has the reservations that 5α-androstane-3β,17β-diol was only tested at one dose and that its distribution and metabolism are largely unknown.

A striking feature of the present results was the difference in activity between the two isomers of 5α-androstanediol which differ only in the orientation of

the hydroxyl group at the 3-position. The 3α-isomer arrested the oestrous cycle and caused rapid tumour regression, whereas the corresponding 3β-hydroxysteroid, given at the same dose, was without significant effect on either parameter. A similar phenomenon was observed with the transplanted mammary fibroadenoma model where different series of steroids with 3-keto and 3α-hydroxyl groups showed a much greater ability to induce regression of both mammary tumours and ovaries than their 3β-hydroxylated counterparts (Huggins ε Mainzer, 1957).

Certain features of the metabolism of 5α-reduced steroids may have a bearing on the differences observed. 5α-Dihydrotestosterone is metabolised to 5α-androstane-3α,17β-diol in preference to the 3β-isomer by most tissues and it has been shown that the former conversion but not the latter is readily reversible in the rat prostate and pituitary (Baulieu, Le Goascogne, Groyer, Feyel-Cabanes ε Robel, 1975; Pilven, Thieulant, Ducouret, Sampérez ε Jouan, 1976; Plasse, Revol ε Lisboa, 1977). The results of the time-course studies in this thesis suggest that this pattern of metabolism also occurs in the DMBA-induced mammary tumour. If the female rat pituitary and DMBA-induced mammary tumour are capable of interconverting 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol but incapable of transforming 5α-androstane-3β,17β-diol to either of the former steroids, it is possible that the 3α-hydroxylated, but not the 3β-hydroxylated, steroid could exert its action at pituitary or tumour level via the production of 5α-dihydrotestosterone. This assumes

that 5α-dihydrotestosterone is the active form required, but the same argument would hold if in fact 5α-androstane-3α,17β-diol is the ultimately active steroid.

The relative importance of direct and indirect effects in androgen-induced inhibition of tumour growth could be largely answered by administering a series of androgens over a range of doses to tumour-bearing rats, in which tumour growth rate could be related to changes in plasma levels of prolactin and ovarian hormones throughout treatment and recovery periods.

Based on the observations that androgens cause regression of DMBA-induced mammary tumours (Heise ε Gorlich, 1966), and that these tumours contain 5α-reductase (King, Gordon ε Helfenstein, 1964; Miller, Forrest ε Hamilton, 1974), an enzyme which is implicated in the expression of many of the effects of testosterone on its target tissues (King ε Mainwaring, 1974), it was hypothesised that the levels of tumour 5α-reductase activity might influence mammary tumour growth by controlling the conversion of testosterone to its active metabolites capable of inhibiting mammary tumour growth. To test this hypothesis, and to examine the possibility that some of the effects of hormones on tumour growth might be mediated by alterations in tumour 5α-reductase activity, tumour growth and 5α-reductase activity were measured in rats subjected to different hormonal manipulations. According to the hypothesis, it was expected that 5α-reductase activity should decrease in tumours whose growth was stimulated and should increase in regressing tumours.

With the assumption that the relative capacities of tumours to perform 5α-reduction in vitro reflected the relative rate of formation of 5α-reduced products in vivo, the changes observed in 5α-reductase activity were the opposite of what was predicted - namely that the faster growing tumours of perphenazine-treated animals in fact exhibited higher 5α-reductase activity than control tumours. It is therefore necessary to examine the basis of the hypothesis in more depth and to examine the strength of the relationship between in vitro and in vivo 5α-reductase activity.

Although the observations of androgen-induced tumour regression and the presence of tumour 5α-reductase are without dispute and were readily confirmed in the present studies, the following fundamental assumptions must also hold for the validity of the hypothesis that tumour 5α-reductase activity influences tumour growth by the local conversion of testosterone to its 5α-reduced metabolites.

1. The ability of testosterone to inhibit mammary tumour growth is mediated by its conversion to 5α-reduced products at tumour level.
2. In the female rat there are sufficient levels of testosterone or other androgenic precursors available which, after local 5α-reduction, could induce tumour regression.
3. The critical factor controlling the action of testosterone at tumour level is 5α-reductase activity and not substrate availability or receptor protein concentration.

With regard to the first assumption the present studies have illustrated that the two major 5α-reduced products of testosterone metabolism by DMBA-induced mammary tumours, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol, are very effective inhibitors of mammary tumour growth. Whilst this demonstrates the capability of 5α-reduced metabolites to induce tumour regression, it has yet to be proven that 5α-reduction of testosterone is obligatory for tumour regression and that 5α-reduced metabolites formed at other sites do not play a role in tumour regression. Furthermore the results of the present study have raised the possibility that androgen-induced tumour regression may be due, at least in part, to a reduction in the secretion of both oestradiol and prolactin. Until it can be conclusively demonstrated that androgens can induce tumour regression without altering the levels of other hormones, the first assumption cannot be made.

The second assumption is basically concerned with the comparison between the levels of androgen precursor in the normal female rat and the levels achieved following the administration of a minimally effective dose of androgen. In the normal female rat peripheral plasma levels of testosterone vary from about 80-90 pg/ml in oestrus and metoestrus to about 170-240 pg/ml in proestrus (Dupon ε Kim, 1973; Rosenfeld, Jones, Dupon, Fang ε Anderson, 1975; Terranova, Saidapur ε Greenwald, 1980). Dupon and Kim (1973) reported that plasma levels of 4-androstenedione, a potential precursor of testosterone,

were similar or slightly above those of testosterone throughout the cycle, whereas Terranova, Saidapur and Greenwald (1980) found higher peak levels of 1.4 ng/ml in proestrus. DHA and its sulphate are present only in low concentrations in the circulation of the rat (Cutler, Glenn, Bush, Hodgen, Graham ε Loriaux, 1978). The milligram quantities of androgen which require to be administered to observe tumour regression (Huggins, Briziarelli ε Sutton, 1959; Griswold, Skipper, Laster, Wilcox ε Schabel, 1966; Heise ε Gorlich, 1966) will almost certainly have resulted in far higher levels of androgen than those found in the normal rat. However, there appears to be no data on the critical blood levels of androgen required for inhibition of tumour growth.

Although the rat prostate and the DMBA-induced rat mammary tumour show opposite responses to androgen treatment it is useful to draw an analogy between the two for consideration of the third assumption. On the basis of the high levels of 5α-reductase activity and co-factor availability in the rat prostate and the plasma levels of testosterone (2-5.5 ng/ml) in the male rat (Corpéchot, Eychenne ε Robel, 1977), it was reasoned that the availability of testosterone in the cell and not the level of 5α-reductase activity was critical for the expression of androgenic effects of testosterone on the prostate (Frederiksen ε Wilson, 1971). Incubation of testosterone with homogenates of DMBA-induced mammary tumour under the standard conditions of the present study resulted on average in a little less than half of the precursor

undergoing 5α-reduction. This represents an enzyme activity of approximately 350 ng/0.5 g homogenate/hour, which is approximately equivalent to 250 pmoles/100 mg homogenate/hour. This value, which is an underestimate of the initial rate of reaction, is approximately half that quoted for the rat prostate, is similar to that of the rat epididymis and is higher than that of homogenates from a variety of other tissues (Gloyna & Wilson, 1969). In view of the extent of 5α-reduction by the tumour incubated without the addition of an NADPH-generating system and the demonstration in DMBA-induced mammary tumours of enzymes required for NADPH production (Hilf, Goldenberg, Gruenstein, Meranze & Shimkin, 1970; Cohen & Hilf, 1974), it seems likely that endogenous co-factors are also readily available in the DMBA-induced mammary tumour. Plasma levels of testosterone in female rats (80-240 pg/ml) are much lower than those given above for the male rat (Dupon & Kim, 1973; Rosenfeld, Jones, Dupon, Fang & Anderson, 1975; Terranova, Saidapur & Greenwald, 1980). Although there is insufficient information available to draw comparisons between the uptake and concentrations of testosterone in the two tissues it would appear that the total 5α-reductase capacity of the DMBA-induced mammary tumour is excessive in comparison with the quantity of testosterone available for metabolism.

However, as mentioned earlier, progesterone and testosterone serve as common substrates, and compete for metabolism by 5α-reductase in several tissues (Frederiksen & Wilson, 1971; Hsia, 1971; Saksena, Lau & Chang, 1976;

Eckstein ε Nimrod, 1977), including the DMBA-induced mammary tumour (Mori, Tominaga ε Tamaoki, 1978; Tamaoki, Mori, Kitamura ε Tominaga, 1978). Moreover it has been reported that the enzyme has a preference for progesterone over testosterone (Frederiksen ε Wilson, 1971; Eckstein ε Nimrod, 1977; Mori, Tominaga ε Tamaoki, 1978). In the female rat, progesterone levels which vary from about 10-50 ng/ml (Piva, Gagliano, Motta ε Martini, 1973; Shaikh ε Shaikh, 1975; Brown, Courtney ε Marotta, 1976; Miller ε Riegle, 1980) are at least 100 times those of testosterone quoted above. The relative concentrations of testosterone and progesterone in the tumour, which are more pertinent to the discussion, cannot be extrapolated from plasma levels because the two steroids may differ in their binding to carrier proteins in blood and in their rate of uptake into the tumour. Nevertheless, the extent of the difference between plasma levels of testosterone, the likelihood that progesterone will be readily taken up into the tumour (progesterone receptor levels are high in the cytoplasm of DMBA-induced mammary tumour (Asselin, Melançon, Moachon ε Bélanger, 1980), and the concentration of progesterone in normal rat mammary gland was reported to be approximately 20 times greater than the corresponding serum concentrations (Wynn, Harris ε Chatterton, 1976)), and the preference of 5α-reductase for progesterone, suggest that only a fraction of the 5α-reductase capacity measured in vitro is available for the 5α-reduction of testosterone in vivo. From the data presently avail-

able it is not possible to determine the extent of the limitations, if any, imposed by progesterone on the 5α-reduction of testosterone available to the tumour in vivo.

Several imponderable factors surround any discussion of the adequacy of receptor levels for mediating androgen-induced tumour regression. Although the degree of 5α-dihydrotestosterone binding to cytoplasmic receptor protein of DMBA-induced mammary tumours is lower than that of other steroids (Asselin, Melançon, Moachon ε Bélanger, 1980), it is not known whether androgen-induced tumour regression is mediated by attachment to this receptor or to another, such as the oestradiol receptor; nor is anything known about the degree of binding required for such an effect. More importantly, it has yet to be conclusively demonstrated that androgen-induced tumour regression is, in fact, mediated by attachment to any form of receptor in the DMBA-induced mammary tumour.

If the assumptions listed above were valid and 5α-reduction of testosterone by tumours played a regulatory role in tumour growth certain relationships should naturally follow. The level of 5α-reductase activity should be directly related to androgen sensitivity and inversely related to tumour growth and inhibitors of 5α-reductase should stimulate tumour growth. The relationship between 5α-reductase activity and androgen sensitivity was not included in the present study. However, although perhaps not directly comparable, the hormone independent transplantable mammary tumour TG 5, derived originally from a DMBA-induced tumour, shows

extremely high 5α-reductase activity (Miller, Stewart ε Hawkins, 1979), but appears to be unresponsive to either testosterone or 5α-dihydrotestosterone (Appendix II). It was found that in transplantable mouse mammary tumours, androgen dependency is more closely related to the capacity for androgen binding in the cytoplasm, than to tumour 5α-reductase activity (Yamaguchi, Kasai, Minesita, Kotoh ε Matsumoto, 1974). No relationship between 5α-reductase activity and rate of tumour growth was observed in any of the five treatment groups or in untreated rats killed at different stages of the oestrous cycle. The effect of inhibitors of 5α-reductase on the growth of DMBA-induced mammary tumours does not appear to have been studied. In such a study the effect of 5α-reductase inhibition in tissues other than the DMBA-induced tumour would have to be considered.

Taken as a whole it is apparent that much more information is required, in order to determine if the 5α-reduction of testosterone by DMBA-induced mammary tumours is important in relation to tumour growth. In particular three fundamental questions require to be answered:- Are androgen levels in the female rat sufficient, if converted to an active form, to induce tumour regression? What is the rate of 5α-reduction of testosterone by the tumour in vivo in relation to blood and tissue levels? How do androgens exert their inhibitory effect on tumour growth?

With regard to in vitro measurements of 5α-reductase activity as a reflection of the relative levels of 5α-

reduction occurring in vivo certain reservations must be made. If co-factor availability restricts 5α-reductase activity in vivo, then alterations in the production of co-factors would alter 5α-reductase activity in vivo, without necessarily affecting the in vitro measurement of 5α-reductase activity in the presence of an added NADPH-generating system. Ovariectomy has been reported to decrease, and the administration of oestradiol or prolactin to increase, the activity of glucose-6-phosphate dehydrogenase which converts $NADP^+$ to NADPH (Dao, 1964; Lerner, 1964; Wiest ε Kidwell, 1969; Hilf, McDonald, Sartini, Rector ε Richards, 1972).

It is possible that without altering 5α-reductase activity the 5α-reduction of testosterone may be specifically affected by alterations in its access to the enzyme. It has been shown that prolactin can stimulate the uptake of testosterone into accessory sex glands (Grayhack ε Lebovitz, 1967; Resnick, Walvoord ε Grayhack, 1974), and progesterone may compete with testosterone for cellular uptake mechanisms (Orestano, Altwein, Knapstein ε Bandhauer, 1975; Smethurst ε Williams, 1975). As already mentioned progesterone can also inhibit the access of testosterone to 5α-reductase by direct competition for the enzyme.

These effects on 5α-reductase activity and on the 5α-reduction of testosterone in particular occur through hormonal changes and therefore, if occurring also in the DMBA-induced tumour, may have been more marked in one treatment regime than in another. However the method used

to measure 5α-reductase activity capacity in vitro would probably obscure such changes. It is plausible that although the total capacity for 5α-reduction was higher in tumours from perphenazine-treated rats the net 5α-reduction of testosterone in vivo was lower because of increased competition for uptake and metabolism from raised levels of progesterone in the circulation in these animals. It has been suggested that in the rat ovary high local concentrations of progesterone and 20α-hydroxy-4-pregnene-3-one may protect against the metabolism of testosterone and 4-androstenedione (Koninckx, Verhoeven, Heyns ε de Moor, 1979).

The possibility of progesterone involvement in the control of 5α-reductase activity in the DMBA-induced rat mammary tumour has already been discussed. If progesterone does, in fact, increase 5α-reductase activity and is the major substrate for the enzyme, it might be worthwhile investigating whether the enzyme level is controlled by a form of substrate induction.

No information could be found regarding the effect of 5α-reduced metabolites of progesterone on DMBA-induced mammary tumours. Although it is generally considered that, for progestational activity, progesterone itself is in a biologically active form (Wiest, 1971), 5α-reduced metabolites have been reported to bind to progesterone receptors (Pollow, Lübbert, Boquoi, Kreuzer ε Pollow, 1975; Coty, Schroder ε O'Malley, 1979), and have been implicated in some of the biological actions of progesterone (Armstrong ε King, 1971).

When this thesis was begun there were already several reports in the literature concerning factors which play a regulatory role in the induction of rat mammary tumours by DMBA. Factors studied included hormonal manipulation, age at DMBA administration and strain difference (Welsch ε Nagasawa, 1977). However, no report appeared to have been published on factors which might be associated with tumour incidence within a colony of untreated rats, all given DMBA at a similar age. It was therefore proposed that in addition to the main study, the stage of oestrous cycle and plasma prolactin levels at the time of DMBA administration should be recorded and related to subsequent tumour incidence. Because this study was of secondary importance and the tumours developed were required for other purposes, certain limitations had to be imposed on the measurements taken. The stage of the oestrous cycle was assessed from a single vaginal smear and because several rats were given DMBA consecutively stress-induced alterations in prolactin may have occurred. The only tumour parameter which could be measured was the presence or absence of tumours within 120 days of DMBA administration. Since it is possible for first tumours to arise beyond this period, this measurement can be considered as a combination of incidence and latency. Although not all of the tumours recorded were examined histologically, it has been reported that non-adenocarcinomatous tumours, such as fibroadenomas and nodular adenoses, have a long latent period and are not usually seen within the observation

period used in this study (Gruenstein, Meranze, Thatcher ε Shimkin, 1966). This agrees with the findings in the tumours which were examined histologically, namely, that nearly all tumours were classified as adenocarcinoma. One strong point of the study was the large number of animals included.

Two significant observations were made. The first was that the distribution of rats over the different stages of the cycle at the time of DMBA administration was different between those which subsequently bore tumours and those which did not. This difference was due to the number of tumour-bearing rats being lower than expected in the group which was in dioestrus at the time of carcinogen administration and being higher than expected in the group which was in proestrus. The second observation was that tumour-bearing rats had higher levels of plasma prolactin at the time of DMBA administration.

As reviewed in the INTRODUCTION the common link between factors which affect tumour induction appears to be the rate of DNA synthesis in the mammary gland at the time of DMBA administration. The susceptibility of the mammary gland to carcinogenic attack is greatest when DNA synthesis is highest (Nagasawa ε Yanai, 1974; Janss ε Hadaway, 1977), and in particular during the period of differentiation of terminal end buds, the structures from which the tumours arise (Russo ε Russo, 1978 b). In rats of 50 days of age, overall DNA synthesis in the mammary gland was found to be fourfold higher in proestrus

than in dioestrus (Nagasawa, Yanai ε Taniguchi, 1976), and it was recently stated that DNA synthesis in the terminal end bud structures of 55-day-old rats was high in proestrus and early oestrus and low in dioestrus (Dulbecco, 1980).

Since the stages of the oestrous cycle in which high and low DNA synthesis occurs are the stages in which DMBA administration resulted in a high and low tumour incidence respectively it is tempting to conclude that the two factors are related. This conclusion is only valid if the initial step in the carcinogenic action of DMBA occurs shortly after it is administered. Indeed it has been demonstrated that peak levels of DMBA were found in the mammary gland 6-16 hours after intragastric administration (Gammal, Carroll, Muhlstock ε Plunkett, 1965; Wieder, Thatcher ε Shimkin, 1967), and that 16 hours after feeding ^{3}H-DMBA a high amount of radioactivity was bound to DNA of parenchymal cells of the mammary gland (Janss, Moon ε Irving, 1971). When pieces of mammary gland were transplanted from rats given DMBA to untreated recipients, tumour incidence was highest in the grafts removed when DMBA content was highest (Dao, King ε Gawlak, 1968).

However the clearance of DMBA or its metabolites from the mammary gland is slow and trace amounts remain in the tissue several days after administration (Wieder, Thatcher ε Shimkin, 1967; Dao, King ε Gawlak, 1968). The compound N-nitroso-N-methylurea, which has recently been reported to induce breast cancer in rats in a sim-

ilar manner to DMBA, but which can only be detected in mammary tissue for a short time after its administration, may offer a superior approach to this question of the variation in susceptibility to carcinogenesis throughout the oestrous cycle (Gullino, Pettigrew ε Grantham, 1975).

While these data were being collected a report on the same subject was published in which DMBA was given to 37 rats in proestrus and 27 rats in dioestrus (Nagasawa, Yanai ε Taniguchi, 1976). Over a follow-up period of 26 weeks no significant difference in the mammary tumour incidence between the two groups was found but the percentage of progressive tumours, growth rate and the number and the weight of tumours per tumour-bearing rat were significantly higher in those given DMBA at proestrus than in animals given it at dioestrus. The lack of noticeable effect on incidence is not surprising since it was extremely high, between 95 and 100% in both groups. Despite the differences between the study of Nagasawa, Yanai and Tanaguchi (1976) and the one presented in this thesis both arrive at the same conclusion, that the susceptibility of the mammary gland to the carcinogenic effects of DMBA is greater when the carcinogen is given in proestrus than when given in dioestrus.

Irrespective of the stage of oestrous cycle, the levels of plasma prolactin were higher in rats which subsequently developed tumours than in those which did not. It has been reported that 'normal' prolactin levels are required at the time of DMBA administration for the

development of tumours (Nagasawa, Chen ε Meites, 1973), and that a reduction in plasma prolactin levels by hypophysectomy (Huggins, Grand ε Brillantes, 1959), and by ergot alkaloids (Clemens ε Shaar, 1972; Nagasawa, Yanai ε Taniguchi, 1976) led to a decreased incidence of mammary tumours. Plasma prolactin levels have also been found to be lower in strains of rats which have a lower tumour incidence (Boyns, Buchan, Cole, Forrest ε Griffiths, 1973; Hawkins, Drewitt, Freedman, Killen, Jenner ε Cameron, 1976). (The difference in incidence between strains could however be due to the levels of plasma prolactin being too low to support tumour growth. It would be interesting to artificially raise plasma prolactin before and/or after DMBA administration in a strain exhibiting abnormally low tumour response to carcinogens.) However the present report appears to be the first to indicate that within a single colony of unmanipulated rats tumour incidence is related to plasma prolactin levels at the time of DMBA administration.

Both in vivo and in vitro studies have shown that prolactin plays an important role in the development of the mammary gland and that it has a stimulatory effect on the role of DNA synthesis by this tissue (Lyons, Li ε Johnson, 1958; Meites ε Nicoll, 1966; Dilley, 1971; Hallowes, Wang ε Lewis, 1973; Stoudemire, Stumpf ε Sar, 1975). It is possible therefore that the rats which bore tumours were more susceptible to a carcinogenic attack than their non-tumour-bearing counterparts because of a greater DNA synthesis rate in their mammary

glands caused by higher prolactin levels at the time of DMBA administration. A correlation between the rate of DNA synthesis and plasma prolactin levels at each stage of the oestrous cycle would have to be demonstrated to support this possibility.

An alternative possibility which was not investigated was that the rats which developed tumours may have continued to exhibit higher prolactin levels than non-tumour-bearing rats after DMBA administration. If this were the case then relationships between plasma prolactin levels and tumour incidence may have resulted from a stimulatory effect of prolactin on development as well as induction of tumours.

Attempts to relate the results of the present study on the DMBA-induced rat mammary tumour to the human situation are hindered by differences between both the properties of human and rat mammary tumours and the endocrinology of the respective host species.

Although both human breast cancer and the DMBA-induced tumour convert testosterone to 5α-dihydrotestosterone and 5α-androstanediol, the ability to do so is greater in tumours of the rat (Miller, Forrest ε Hamilton, 1974). In view of the observation that 5α-dihydrotestosterone is a potent inhibitor of the aromatisation system (Scharrzel, Kruggel ε Brodie, 1973; Siiteri ε Thompson, 1975; Hillier, van den Boogaard, Reichert ε van Hall, 1980), the higher production of this steroid by rat mammary tumours in vitro may account for the fact that oestradiol synthesis has never been conclusively

demonstrated in the DMBA-induced mammary tumour, whereas it has been demonstrated in approximately half of the human tumours (Li, Chandra, Foo, Adams ε McDonald, 1976; Miller ε Forrest, 1976; Abul-Hajj, Iverson ε Kiang, 1979 a). Much of the metabolism of testosterone by human mammary tumours remains unaccounted for. It may be worthwhile investigating the formation of steroid allyl alcohols which has now been shown to take place in the DMBA-induced tumour.

In relation to the stimulation of tumour 5α-reductase by administration of perphenazine to rats it is interesting that exceptionally high 5α-reductase activity has been observed in a small number of breast cancers from patients who had been receiving the prolactin-releasing drug, methyl-dopa, for some time (Miller, personal communication). Whereas it was possible to postulate that in the rat the increase in 5α-reductase activity could be mediated by a stimulation in plasma progesterone levels, prolactin does not appear to perform the same luteotrophic role in the human (Nicoll, 1974). However it may be relevant that chronically elevated levels of plasma prolactin have been associated with an increased secretion of C-19 androgen precursors from the adrenal of postmenopausal women (Ando ε Vermeulen, 1977; Murru, Romagnino, Genazzani ε Fioretti, 1977; Vermeulen, Suy ε Rubens, 1977). It would be of interest to know if there was a relationship between their plasma levels and tumour 5α-reductase activity.

On account of the high level of 5α-reductase in the DMBA-induced mammary tumour and the relatively low levels of circulating C-19 steroids in the female rat, the role of 5α-reductase in controlling the action of androgens on this tumour has been questioned. However, the higher levels of C-19 steroids in the blood of women and the relatively lower tumour 5α-reductase activity mean that the level of 5α-reductase may be of more significance in controlling the sensitivity of human breast cancer to androgens.

SUMMARY

In common with certain human breast cancers, rat mammary adenocarcinomas induced by DMBA regress on the administration of pharmacological doses of androgen. Mammary cancers of both species convert testosterone by 5α-reduction to 5α-dihydrotestosterone and 5α-androstanediol. This conversion plays an important role in mediating the androgenic effects of testosterone in target organs. The relationship between plasma hormone levels, tumour growth and metabolism of testosterone by DMBA-induced tumours has been investigated in rats of differing endocrine status.

Female Sprague-Dawley rats bearing actively growing tumours, induced by a single intragastric administration of 30 mg DMBA at 50 days of age, were allocated to one of the following treatment groups:- 1) Intact Control, 2) Intact + Perphenazine, 3) Intact + CB 154, 4) Ovariectomised Control, 5) Ovariectomised + Perphenazine. Animals received a daily s.c. dose of drug (5 mg/kg body weight) or corn oil vehicle (control groups) over an average treatment period of 12 days. Tumour size and the stage of the oestrous cycle, assessed from daily vaginal smears, were monitored before and during treatment. The effects of the treatment regimes on plasma prolactin and oestradiol levels, the two hormones considered of major importance in the growth of DMBA-induced mammary tumours, were measured in individual tumour-bearing rats and also in greater detail in parallel studies in non-tumour-bearing rats. At the end of treatment (on the day of dioestrus in cycling animals)

a portion of tumour (0.5 g) was minced, sonicated and incubated for one hour at 37°C with 50 x 10^6 d.p.m. [7α-^{3}H]testosterone in Krebs-Ringer phosphate buffer, pH 7.4, containing an NADPH-generating system. Steroid interconversions were determined by measuring radioactive label in individual metabolites after extraction and purification by thin layer chromatography.

Under these conditions tumour preparations from the intact control group metabolised slightly more than half of the testosterone precursor, mainly by 5α-reduction (determined as the sum of 5α-dihydrotestosterone and 5α-androstanediol production). The major isomer of 5α-androstanediol was the 3α,17β-diol. In comparison with the intact control group, the administration of perphenazine to intact rats raised plasma prolactin levels, lowered plasma oestradiol levels, arrested the oestrous cycle in dioestrus and stimulated mammary tumour growth. Tumours in this group showed a greater capacity for testosterone metabolism and had significantly elevated levels of 5α-reduction. Administration of CB 154 depressed plasma prolactin levels but did not decrease plasma oestradiol levels, nor did it arrest the oestrous cycle. In these rats tumour growth was, in general, inhibited but the metabolism of testosterone did not differ significantly from that of the intact control group. Ovariectomy caused a fall in plasma oestradiol and plasma prolactin levels, a constant dioestrus-type vaginal smear, and rapid tumour regression. Although the mean level of 5α-reduction in tumours from

this group was numerically lower than that of the intact control group, the difference did not attain significance. Plasma prolactin levels in ovariectomised rats given perphenazine were elevated to the same extent as in intact rats given perphenazine but tumour growth was not maintained. Whilst the level of 5α-reduction in tumours from ovariectomised rats given perphenazine was significantly higher than in tumours from the ovariectomised control group, it did not differ significantly from that of tumours from the intact control group and was significantly lower than that of tumours from intact rats given perphenazine. It is concluded that tumour growth is largely dependent on plasma prolactin levels and on the presence of ovaries. Although tumour 5α-reduction was increased in rats with elevated plasma prolactin levels other intergroup comparisons indicate that ovarian hormones could also be involved in the regulation of tumour 5α-reductase activity. In general there was no consistent correlation between tumour growth rate and tumour 5α-reductase activity.

A study of testosterone metabolism in actively growing DMBA-induced mammary tumours, taken from untreated rats at different stages of a 4-day oestrous cycle, revealed that 5α-reductase was highest in tumours removed in metoestrus and lowest in tumours removed in proestrus. However the differences in 5α-reduction levels in tumours from the four stages of the cycle did not attain significance. Of the other parameters measured only plasma oestradiol levels varied signifi-

cantly throughout the cycle, the level being greatly raised in proestrus.

DMBA-induced mammary tumours appear to possess an extremely high capacity for 5α-reduction in relation to circulating levels of androgen precursor in the female rat. On the other hand progesterone, which has been reported to be a preferred substrate to testosterone for 5α-reductase, is present in relatively high concentrations in the plasma of female rats. The possibility that an important function of tumour 5α-reductase in vivo may be the conversion of progesterone to 5α-reduced products is therefore raised.

Evidence is presented for the conversion of testosterone to androst-4-ene-3α,17β-diol by DMBA-induced rat mammary tumours. The formation of this metabolite appeared to be inversely related to the formation of 5α-reduced products and was responsible for a large part of the metabolism of testosterone otherwise unaccounted for by 5α-reduction.

The daily administration (1 mg s.c./rat) of 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol for about 12 days resulted in constant dioestrus-type smears and profound regression of DMBA-induced mammary tumours. In contrast, the same dose of 5α-androstane-3β,17β-diol was without effect on either parameter. Plasma prolactin levels in the two groups of non-cycling, androgen-treated rats were similar to those of control cycling rats in dioestrus. It is therefore plausible that, in addition to a direct effect at tumour level,

androgen-induced tumour regression may be mediated by an inhibition in the secretion of oestradiol and prolactin.

In a total of 861 rats, the plasma prolactin level and the stage of the oestrous cycle at the time of DMBA administration were related to subsequent tumour appearance over an observation period of 120 days. Tumour incidence was higher in rats given DMBA in proestrus, and lower in those given DMBA in dioestrus. At all stages of the cycle the mean plasma prolactin levels at the time of DMBA administration were higher in rats which subsequently bore tumours than in those which did not. These observations may reflect a relationship between the rate of cell division in components of the breast and susceptibility to carcinogenic attack.

APPENDICES

Appendix I

Testosterone metabolism and oestrogen receptor levels

Some of the tumours which were incubated for testosterone metabolism studies were also assayed for their oestradiol receptor content in Dr R. A. Hawkins' laboratory. Although some of the oestrogen receptor results have already been published (Hawkins, Hill, Freedman, Killen, Buchan, Miller & Forrest, 1977), no data on testosterone metabolism were included. In Table 39 the percentage 5α-reduction of testosterone is given along with the oestrogen receptor content for all tumours on which both assays were performed. The dissociation constant (K_d) values of the oestrogen receptors in these tumours ranged from 0.08 - 0.86 x 10^{-10} M. Since the assay measured unoccupied cytoplasmic binding sites whose levels may be affected by the levels of oestradiol in the circulation (Hawkins, Hill, Freedman, Killen, Buchan, Miller & Forrest, 1977), plasma oestradiol levels are also listed. Further details of rats and tumours are given in the tables in Sections III and IV of the RESULTS.

Although the tumours from intact rats given perphenazine showed high levels of 5α-reduction and had a high oestrogen receptor content there did not appear to be a correlation between these 2 parameters over the small number of tumours within each group. In 5 of the 6 rats from which data on 2 tumours were available, the tumour with highest 5α-reductase activity also had the highest oestrogen receptor content.

Table 39

Oestrogen receptor levels and 5∝-reductase activity in DMBA-induced mammary tumours

Treatment group	Rat and tumour	5∝-reduction of testosterone (%)	Receptor content (Po) (fmol/mg tissue)	Plasma oestradiol (ng/100ml)
dioestrus	45M RIn	52.94	4.93	0.52
I C	343L LNk	43.00	1.68	0.81
	LGr	27.17	1.44	
I + P	309E RNk	81.93	3.13	0.10
	LIn	81.36	2.57	
	318E RAx	41.66	4.41	0.61
	333E RNk	39.80	4.20	0.24
	343E RIn	64.10	2.22	0.92
	357E LAx	37.19	2.57	0.27
	60H LGr	68.53	2.51	0.23
	87H RGr	70.48	6.05	0.28
I + B	345L LAx	36.75	0.82	2.02
	LIn	48.45	1.52	
	351L LTh	54.27	2.07	0.97
	354L LCh	18.89	1.35	1.08
	361L LTh	39.92	1.06	1.24
	LAn	46.47	1.15	
	366L RGr	55.31	1.68	0.60
	384L RAx	41.05	2.49	0.69

Table 39 (continued)

Treatment group	Rat and tumour	5α-reduction of testosterone (%)	Receptor content (Po) (fmol/mg tissue)	Plasma oestradiol (ng/100ml)
O C	306K LNk	15.14	1.10	0.33
O + P	378E RCh	63.57	1.40	0.22
	RAx	32.13	1.55	
	397E RCh	71.54	2.88	0.29
	26H RAx	38.96	0.64	0.06
	297K RAx	38.30	0.92	0.43
	LAx	18.89	0.51	

Appendix II

Effect of androgens on a transplantable mammary tumour

A tumour line, TG-5, which originated from a DMBA-induced mammary tumour, was maintained by serial transplantation into an inbred colony of rats originally purchased from the Animal Diseases Research Association, Edinburgh. With successive transplantations, tumours became ovarian independent and displayed high 5α-reductase activity (Miller, Stewart & Hawkins, 1979). Because of these features the effect of androgens on tumour growth was investigated.

Small pieces of tumour from the 31st transplant generation were implanted s.c. in the backs of 75-day-old rats. Approximately 2 weeks after the appearance of the first tumour, animals were given testosterone or 5α-dihydrotestosterone, dissolved in corn oil, in daily s.c. doses of 5mg/kg. Tumour size was measured 3 times per week and vaginal smears taken daily before and during treatment. Since it was known that all transplant tumours grow continuously no control group was included.

It can be seen from the results in Table 40 that the growth of these tumours was not inhibited by androgen treatment. Vaginal smear patterns in rats of this colony were less regular than those of the rats used for inducing tumours with DMBA. Furthermore the rats of this colony did not exhibit a constant pattern of dioestrus-type vaginal smears during androgen treatment.

Table 40

The effect of androgens on the growth of a transplantable mammary tumour

	Treatment	
	5α-DHT	Testosterone
Number of rats	5	4
Number of tumours	9	9
Pretreatment		
number growing	9	9
size (cm^2)	2.55 ± 0.94	2.54 ± 0.85
growth rate (cm^2/week)	1.38 ± 0.52	1.12 ± 0.41
End of treatment		
number growing	9	9
size (cm^2)	5.75 ± 2.86	6.32 ± 2.07
growth rate (cm^2/week)	1.81 ± 1.28	1.86 ± 0.65

Values of size and growth rate are means $\pm$ s.d.'s.

Each rat received 12 daily s.c. doses of 5mg steroid/kg body weight.

Appendix III

Standardisation of plasma prolactin results

Because of the inevitable deterioration of certain of the reagents used in the prolactin assay, interassay variations could occur. Therefore, from time to time, samples of fresh plasma from male rats were pooled, and divided into aliquots for use as quality controls. These were stored in the same manner as other samples, and duplicates included in each assay. Quality control values were read off the standard curve of each assay, and are listed in Table 41. In order to standardise the results of different assays a reference value for Pool E was fixed arbitrarily at 20ng/ml (after this value had been obtained in 3 consecutive assays), and the sample values obtained from the standard curve of each assay multiplied by the correction factor,

$$\frac{\text{reference value of pool}}{\text{quality control value in assay.}}$$

The reference value for a new pool relative to the original 20ng/ml of Pool E was determined from the average ratio of values of the old and new pools run in the same assay (Table 41).

Table 41

Quality control values and calculation of reference values in prolactin assays

Assay number	Prolactin (ng/ml) Pool E	Pool G	Pool P	
63	21			
64	25			
65	20			Pool E
66	20			ref. value = 20ng/ml
67	20			
69	13			
70	10			
72	18			
73	17			
74	16			
76	24			
77	25			
78	25	62		
79	32	54		
80	27	44		
81	30	60		Ave G/E = 2.12
82	31	56		Pool G
83	25	50		ref. value = 20 x 2.12
84	21	50		
85	19	49		
86	30			
87	24	50		
88	25	55		
89	29	60		
90		53		
91		49		
96		72		
97		74		

Table 41 (continued)

Assay number	Prolactin (ng/ml) Pool E	Pool G	Pool P	
100		53		
101		50	40	
102		43		
103		44		Ave P/G = 0.80
107		40	32	Pool P
108		29	23	ref. value = 42.2 x 0.80
110		30		
111		34	27	
112			23	
113			17	
114			23	
115			29	
116			33	
117			24	

Appendix IV

Statistical analysis

The choice and application of statistical analyses and formulae used in this thesis are described in the publications listed below.

1. Dunnett's test -

   Dunnett, C. W. (1964)

   New tables for multiple comparisons with a control

   Biometrics 20, 482 - 491

2. Table for d_2 in s.d. (est.) $= \frac{\bar{R}}{d_2}$ -

   Grant, E. L. (1952)

   Statistical quality control. 3rd Edition

   New York: McGraw-Hill

3. All other tests -

   Snedecor, G. W. and Cochran, W. G. (1976)

   Statistical methods. 6th Edition

   Iowa State University Press

Appendix V

Publications

Part of the work carried out for this thesis has been described in the following publications:-

Buchan, P., Fraser, A. T. and Miller, W. R. (1976)

The effect of perphenazine treatment on testosterone metabolism by established rat mammary carcinomas.

Biochem. Soc. Trans. 4, 1100 - 1102

Buchan, P. and Miller, W. R. (1978)

Perphenazine and testosterone metabolism by mammary tumours in oophorectomised rats.

Biochem. Soc. Trans. 6, 135 - 136

The Effect of Perphenazine Treatment on Testosterone Metabolism by Established Rat Mammary Carcinomas

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Mammary carcinomas may be induced in female Sprague–Dawley rats by the administration of the carcinogen 7,12-dimethylbenzanthracene. These tumours metabolize testosterone (King *et al.*, 1964), particularly by 5α-reduction to 5α-dihydrotestosterone and 5α-androstanediol (Miller, 1976). In a previous study, tumours obtained from animals rendered hyperprolactinaemic by giving perphenazine during the period of tumour induction and growth showed enhanced conversion of testosterone into 5α-reduced metabolites (Miller *et al.*, 1974*a*). The aim of the present study was to determine if similar effects could be elicited by administering perphenazine to animals with established tumours and raising plasma prolactin concentrations during the growth period alone.

Tumours induced in female Sprague–Dawley rats by intragastric administration of dimethylbenzanthracene (30mg in cotton-seed oil) at 50 days of age were measured three times weekly by calipers. Animals bearing tumours showing continuous growth were allocated to control or treatment groups when the tumour size was about 1.5cm × 1.5cm. The treatment group received daily subcutaneous injections of perphenazine (5mg/kg body weight) until being killed 12 days later. Control animals did not receive the drug over the same time-period. Measurement of tumour size continued during treatment until excision at death. The metabolism of testosterone *in vitro* was then determined in ten tumours from each group. A portion of each tumour (0.5 g) was finely sliced and sonicated in 5ml of Krebs–Ringer phosphate buffer, pH7.4. An NADPH-generating system (100μmol of glucose 6-phosphate, 15μmol of $NADP^+$ and 25 units of glucose 6-phosphate dehydrogenase) and 20μCi of [7α-^{3}H]testosterone were added to give a final volume of 7.5ml. The incubations were then shaken in an atmosphere of O_2 at 37°C for 1h.

The steroid interconversions were determined by measuring the percentage incorporation of radioactive label into the individual metabolites after extraction and purification by t.l.c. Details of the methods of steroid purification and characterization have been described by Miller *et al.* (1974*b*).

When sufficient tumour material was available, the DNA content was measured by the method of Burton (1956).

All but one of the tumours from the perphenazine-treated group showed an acceleration of growth during the treatment period, whereas those from control animals showed no significant change.

The results from the incubations with [7α-^{3}H]testosterone are summarized in Table 1. Tumours from perphenazine-treated animals showed significantly higher metabolism of testosterone than those from control animals. The major metabolites of testosterone in tumours from both control and perphenazine-treated animals were 5α-dihydrotestosterone and 5α-androstanediol. The conversion into both 5α-dihydrotestosterone and 5α-androstanediol was, however, significantly higher in tumours from the perphenazine-treated group. The increase in 5α-reduction alone accounts for the higher concentrations of testosterone metabolized by tumours from this group. These effects of perphenazine are unlikely to be mediated by an increase in tumour cellularity, as the tumour DNA content was similar in both groups of animals.

It is concluded that short-term administration of perphenazine stimulates growth and testosterone metabolism in dimethylbenzanthracene-induced rat mammary carcinomas. Preliminary results show that these changes were accompanied by an increase in plasma prolactin.

Burton, K. (1956) *Biochem. J.* **62**, 315–323
King, R. J. B., Gordon, J. & Helfenstein, J. E. (1964) *J. Endocrinol.* **29**, 103–110
Miller, W. R. (1976) *Br. J. Cancer* **33**, 474–477
Miller, W. R., Buchan, R. & Forrest, A. P. M. (1974*a*) *Biochem. Soc. Trans.* **2**, 312–314
Miller, W. R., Forrest, A. P. M. & Hamilton, T. (1974*b*) *Steroids* **23**, 379–395

Table 1. *Metabolism of [7α-^{3}H]testosterone by carcinomas from perphenazine-treated and control animals*

Results are given as means±S.E.M. for ten tumours, except for * mean for six tumours and † mean for nine tumours. 5α-Reduction (%) is calculated as combined conversion into 5α-dihydrotestosterone and 5α-androstanediol. Significance is calculated by the Wilcoxon Rank test. N.s., Not significant.

	Metabolism of testosterone (%)	Conversion into 5α-dihydrotestosterone (%)	Conversion into 5α-androstanediol (%)	5α-Reduction (%)	DNA content (μg/mg of tissue)
Control	49.07±4.59	5.98±0.98	26.68±4.47	32.66±5.07	6.58±1.42*
Perphenazine-treated	67.65±3.82	17.28±3.54	44.66±2.90	61.94±5.26	7.93±0.88†
Significance	$P<0.01$	$P<0.05$	$P<0.01$	$P<0.01$	N.S.

A-12

Perphenazine and Testosterone Metabolism by Mammary Tumours in Oophorectomized Rats

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Rat mammary tumours induced by the carcinogen 7,12-dimethylbenzanthracene in female rats metabolized testosterone, particularly by 5α-reduction to 5α-dihydrotestosterone and 5α-androstanediol (Miller, 1976). It has also been shown that these conversions were significantly elevated in tumours from animals treated with perphenazine (2-{4-[3-(2-chlorophenothiazin-10-yl)propyl]piperazin-1-yl}ethanol) (Buchan *et al.*, 1976). Additionally, perphenazine stimulated tumour growth, raised plasma prolactin concentrations and interrupted the normal oestrous cycle. To determine the relative influence of prolactin and ovarian hormones on tumour growth and steriod metabolism, the effects of perphenazine have been studied in oophorectomized animals.

Female Sprague–Dawley 50-day-old rats were given 7,12-dimethylbenzanthracene (30 mg in cottonseed oil) intragastrically. Tumours subsequently induced were measured three times weekly by calipers throughout the study. Animals bearing tumours that showed continuous growth were allocated to the treatment or the control group when the tumour size exceeded 2 cm × 2 cm. The allocation was performed on the day of dioestrus when all animals were bled from the tail vein and bilaterally oophorectomized. The treatment group of rats additionally received daily subcutaneous injection of perphenazine (5 mg/kg body wt.) from the day of oophorectomy until death 14 days later. Control animals received daily doses of the injection vehicle (corn oil) over the same period. Immediately before death, blood was taken for plasma prolactin determination by radioimmunoassay, and tumours taken for steroid metabolism studies *in vitro*. Ten tumours from both animal groups were investigated: 0.5 g of each tumour was finely sliced and incubated at 37°C for 1 h in 5 ml of Krebs–Ringer phosphate buffer, pH 7.4, containing 20 μCi of [7α-^{3}H]testosterone and NADPH-generating system (Buchan *et al.*, 1976). Methods for purification and characterization of testosterone and its metabolites have been described in detail previously (Miller *et al.*, 1974).

The effects of the treatment regimes on plasma prolactin concentrations are shown in Table 1. Both groups of animals had similar prolactin values at the time of allocation. However, whereas oophorectomy alone resulted in a fall in plasma prolactin concentration in each animal, oophorectomy in combination with perphenazine treatment produced an increase in prolactin in all animals. The difference between pre- and post-treatment values was significantly for both animal groups.

Oophorectomy alone produced an immediate regression of all tumours. In the oophorectomy-plus-perphenazine group, however, there was a variable response in tumour growth, certain tumours increasing in size throughout the time of study whereas others regressed, usually after a short period of continued growth.

The metabolism of [7α-^{3}H]testosterone by tumours from both groups of animals is shown in Table 2. Tumours from animals given perphenazine in addition to oophorect-

Table 1. *Effect of oophorectomy and subsequent perphenazine administration on plasma prolactin concentrations*

Animal group	Plasma prolactin (ng/ml) (means + S.E.M.) Before treatment	After treatment	Significance*
Oophorectomy alone	37.1 ± 11.4	6.7 ± 1.0	$P<0.01$
Oophorectomy and perphenazine administration	37.3 ± 10.8	87.9 ± 10.6	$P<0.01$

* Significance was measured by paired Student's *t* test.

Table 2. *Metabolism of [7α-^3H]testosterone by tumours from the animal groups studied*

Significance, by Wilcoxon–Rank test, of perphenazine-treated animals paired with oophorectomy-alone group for each conversion was: ‡, not significant; †, $P<0.1$; *, $P<0.05$.

Animal group	Metabolism of testosterone (%)	Conversion into 5α-dihydrotestosterone (%)	Conversion into 5α-androstanediol (%)	5α-Reduction (%)
Oophorectomy alone	40.09 ± 5.43	4.53 ± 0.66	22.96 ± 3.40	27.49 ± 3.74
Oophorectomy and subsequent perphenazine administration	57.65 ± 4.83*	8.97 ± 2.32‡	33.22 ± 4.43‡	42.17 ± 6.13†

omy showed significantly higher metabolism of testosterone than tumours from rats subjected to oophorectomy alone. In all tumours, irrespective of the animal group from which they were derived, the major metabolites of testosterone were 5α-dihydrotestosterone and 5α-androstanediol. Although the mean conversions into both metabolites were greater in tumours from animals given perphenazine, the differences from the oophorectomy alone-group were not significant.

The present results contrast with effects in intact animals where perphenazine, although producing a similar elevation in plasma prolactin values, stimulates tumour growth and causes a highly significant increase in tumour 5α-reduction of testosterone (Buchan *et al.*, 1976).

In summary, perphenazine failed to reproduce, in animals deprived of ovarian hormones, the marked stimulatory effects on mammary-tumour growth and steroid metabolism that it produces in endocrine-intact animals.

Buchan, P., Fraser, A. T. & Miller, W. R. (1976) *Biochem. Soc. Trans.* **4**, 1100–1102
Miller, W. R. (1976) *Br. J. Cancer* **33**, 474–477
Miller, W. R., Forrest, A. P. M. & Hamilton, T. (1974) *Steroids* **23**, 379–395

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